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(54) Title: FLUORESCENT POLARIZATION DETECTION IN MICROFLUIDIC SYSTEMS

(57) Abstract

Fluorescent polarization assays are used in microfluidic systems to perform molecular binding assays and to screen for potential affectors of the molecular binding functions. The fluorescent polarization assays are performed in an entirely homogeneous assay format and are generally performed in a continuously flowing channel of a microfluidic device.

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FLUORESCENT POLARIZATION DETECTION IN MICROFLUIDIC SYSTEMS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application is a regular application claiming priority to Provisional Patent Application Nos. 60/088,650, filed June 9, 1998, 60/101,040, filed September 18, 1998, and 60/118,599, filed February 4, 1999. The disclosure of each of these applications is incorporated herein by reference in its entirety for all purposes.

BACKGROUND OF THE INVENTION

In the analysis of biological and chemical systems, a number of advantages are realized by the process of miniaturization. For example, by miniaturizing analytical and synthetic processes, one obtains advantages in: (1) reagent volumes, where reagents are rare and/or expensive to produce or purchase; (2) reaction times, where mixing or thermal modulation of reactants is a rate limiting parameter; and (3) integration, allowing one to combine multiple preparative and analytical/synthetic operations in a single bench-top unit.

Despite the advantages to be obtained through miniaturized laboratory systems, or microfluidic systems, early attempts at developing such systems suffered from a number of problems. Of particular note was the inability of early systems to control and direct fluid movement through microfluidic channels and chambers in order to mix, react and separate reaction components for analysis. Specifically, many of the early microfluidic systems utilized micromechanical fluid direction system, e.g., microfabricated pumps, valves and the like, which were expensive to fabricate and required complex control systems to be properly operated. Many of these systems also suffered from dead volumes associated with the mechanical elements, which prevented adequate fluid control substantially below the microliter or 100 nanoliter range. Pneumatic systems were also developed to move fluids through microfluidic channels, which systems were simpler to operate. Again, however, these systems lacked sufficient controllability to move small, precise amounts of fluids.

Pioneering developments in controlled electrokinetic material transport have subsequently allowed for the precise control and manipulation of extremely small amounts of fluids and other materials within interconnected channel structures, without the need for mechanical valves and pumps. See Published International Patent Application No. WO

96/04547, to Ramsey. In brief, by concomitantly controlling electric fields in a number of intersecting channels, one can dictate the direction of flow of materials and/or fluids at an unvalved intersection.

These advances in material transport and direction within microfluidic channel networks have provided the ability to perform large numbers of different types of operations within such networks. See, e.g., commonly owned Published International Application No. 98/00231 to Parce et al., and Published International Application No.98/00705, describing the use of such systems in performing high-throughput screening operations.

It would generally be desirable to provide assay systems and methods of broad applicability for use in these improved assay devices and systems, for use in high-throughput discovery processes. The present invention meets these and a variety of other needs.

SUMMARY OF THE INVENTION

The present invention provides methods, apparatus and systems for performing molecular binding assays in a convenient and rapid format. In particular, the present invention provides for the performance of high-throughput screening assays in microfluidic systems using fluorescent polarization detection to assay molecular binding events, and identify potential affectors of those binding functions based upon their effects on the level of fluorescent polarization.

In particularly preferred aspects, the invention provides methods and apparatuses in which continuously flowing assay components are screened against periodically introduced flowing test compounds to determine whether the test compounds have any effect on the binding of the assay components. In more preferred aspects, these assays are carried out in microfluidic devices and systems.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic illustration of an overall system for carrying out the methods of the present invention.

Figure 2 is a schematic illustration of a fluorescent polarization detection system.

Figure 3 is a schematic illustration of one example of a microfluidic device for performing high-throughput screening assays in accordance with the methods of the present invention.

Figure 4 is a schematic illustration of another example of a microfluidic device for performing high-throughput screening assays in accordance with the methods of the present invention.

Figure 5 illustrates a microfluidic device used to demonstrate the efficacy of fluorescence polarization assays in microfluidic systems, as described herein.

Figure 6 illustrates fluorescent intensity data for parallel and perpendicular fluorescence for free labeled ligand, and two different concentrations of unlabeled antiligand.

Figure 7 illustrates fluorescent polarization levels as a function of the antiligand concentration.

Figure 8 illustrates fluorescent intensity levels for parallel and perpendicular fluorescence for free labeled ligand, a mixture of labeled ligand and unlabeled antiligand and the mixture in the presence of three different concentrations of unlabeled ligand (competitor).

Figure 9 is a graph illustrating the dose response of the fluorescence polarization in the presence of increasing levels of unlabeled ligand.

Figure 10 is a schematic layout of microfluidic device for carrying out the fluorescence polarization binding assay experiments shown in Figures 9-12.

Figure 11 is an example of fluorescence polarization data carried out in an inverted fluorescence microscope.

Figure 12 shows a saturation binding curve for the N1 target protein titrated into a fixed concentration (25 nM) of the labeled probe peptide.

Figure 13 are competition binding curves for the N1 target protein using a known competitor (NL932) and an irrelevant compound (N16914).

Figure 14 is a competition binding curve for the N1 target protein using a known competitor (NL932).

Figure 15 is an illustration of a microfluidic device channel geometry used in performing a fluorescence polarization detection based assay, as described herein.

Figure 16 illustrates data for polarization vs. time in a binding experiment where the labeled peptide is constantly flowing and the sipper alternates between the binding protein and buffer.

Figure 17 illustrates data from a competitive inhibition binding assay using fluorescence polarization detection where the binding protein and labeled peptide are constantly flowing and different concentrations of competitor are being sipped from a microtiter plate.

Figure 18 illustrates the raw intensity data for the parallel and perpendicular components of Figure 17.

DETAILED DESCRIPTION OF THE INVENTION

In general, the present invention provides for the use of fluorescent polarization detection to detect an interaction between two compounds flowing through a microscale channel.

I. High Throughput Screening in Microfluidic Systems

As noted above, microfluidic systems have recently been applied to high-throughput experimentation, and particularly in the area of pharmaceutical discovery experimentation. Biological binding functions, e.g., receptor-ligand binding, antibody-antigen binding, etc., are of particular interest in such screening applications, due to their importance in biological functions, generally, and in the initiation or progression of certain biological disorders in particular. Despite their importance, binding functions can present a number of difficulties in terms of assay format. In particular, in typical assay formats, the binding of one molecular species to another is not accompanied by the creation of a signal, e.g., a fluorescent signal, that is indicative of binding. As used herein, the term "ligand" and "antiligand" or "receptor," are used interchangeably to describe two members of a specifically associating pair of molecular species, e.g., receptors and their ligands, antibodies and their antigens, complementary nucleic acids (including deoxynucleic acids, ribonucleic acids, peptide nucleic acids or other nucleic acid analogs), specifically interacting binding proteins or peptides etc. Although generally described in terms of specifically associating binding pairs, it will be appreciated that the methods described herein are also useful in detecting non-specific associations or associative interactions between two compounds, e.g., purely charged based or hydrophobicity/hydrophilicity based interactions.

For example, when a labeled ligand is contacted with an unlabeled receptor, the result of binding is a labeled complex that, in solution, is indistinguishable from the free, labeled ligand. In order to quantitate binding, it is therefore necessary to determine the amount of label that is supplied by the bound component of the ligand, as distinguished from that supplied by the free component. This is typically carried out by tethering the receptor to a solid support, e.g., a surface, bead or other matrix. The free ligand is then separated from the bound by washing the solid support after binding. The amount of label bound to the support then indicates the level of binding. Alternatively, one can perform a separation

operation on the reaction products whereby the free and bound components are separated in a mobile phase format, e.g., electrophoresis, gel or thin layer chromatography, etc.

While these methods are often suitable for performing small numbers of assays, as can be seen, these methods with their multiple steps are not easily adapted to high-throughput or ultra high throughput screening assay, where thousands, hundreds of thousands or even millions of test compounds are screened in equal numbers for their effects in separate assays.

Commonly owned Published International Application No. 98/00231, to Parce et al., describes, *inter alia*, an approach to performing screening assays in microfluidic systems by continuously flowing assay components through a microfluidic channel using, e.g., electroosmotic flow. Specifically, the assay components, e.g., ligand and receptor, are separately introduced into a microfluidic channel from separate reservoirs that contain the ligand and receptor. The components are then flowed along the main channel, e.g., as a result of an applied electric field along the main channel. The assay components produce a steady state signal level that is indicative of the normal level of the assay components' function, i.e., the control. The test compounds to be screened are introduced serially into the channel and mixed with the assay components. Where a test compound shows a signal level that deviates from the normal signal profile, it is identified as an affector of the particular function being assayed. These continuous flow assay systems permit the serial analysis of large numbers of different compounds in a given assay. Further, the small scale of the assay systems permits these analyses to progress without the use of large amounts of reagents and/or test compounds, as well as permitting the use of highly parallelized assay systems, e.g., multiple analysis channels, to further increase the throughput of the system.

In the case of binding assays, under electrokinetic material transport, bound and free labeled ligand typically move at different velocities through the channel, as a result of their different electrophoretic mobilities in the channel. These different mobilities are then used to differentiate the free, labeled ligand from the bound, labeled ligand. While these methods are very effective in identifying affectors of binding functions, they rely largely upon the assay components having sufficiently different mobilities in the electrokinetic system to permit separate detection. Such separation is also dependent upon the off-rate of the complex being relatively slow in the timescale of the reaction, which is not typically the case for many biological reactions. Specifically, for faster off-rate reactions, the equilibrium of the dissociation reaction for the bound complex is skewed during electrophoretic

separation of the free and bound components, e.g., driving up the dissociation rate as the components separate.

The methods of the present invention, on the other hand, permit a totally homogeneous binding assay system that is readily applied to the above-described microfluidic systems, and that produces a detectable event upon binding of one molecular species to the other, which detectable event does not require any separation steps or further labeling steps. In particular, the methods, devices and systems of the present invention provide for assays where a fluorescently labeled ligand is contacted with an unlabeled antiligand in a flowing microfluidic system. The binding of a relatively small labeled ligand with an unlabeled antiligand produces a typically large fluorescently labeled complex. This binding results in a substantial change in the level of fluorescent polarization emanating from the assay mixture, which change is indicative of and proportional to a level of bound label. This level of fluorescent polarization is then compared to polarization from the same assay performed in the presence of different test compounds in the microfluidic system. Thus, the present invention provides for the use of fluorescent polarization detection in determining an effect of a test compound on an interaction of first and second compounds flowing through a microscale channel.

II. Fluorescence Polarization, Generally

Measurement of differential polarization of free and bound ligands has long been utilized to determine relative ligand binding levels, and even to screen for compounds or conditions that might affect that binding. To date, such assays have been carried out in a contained fluid system, e.g., a cuvette or multiwell plate, where the components of the binding reaction, e.g., a labeled ligand and its receptor, are mixed in the presence or absence of a compound to be tested.

The principles behind the use of fluorescence polarization measurements as a method of measuring binding among different molecules are relatively straight-forward. Briefly, when a fluorescent molecule is excited with a polarized light source, the molecule will emit fluorescent light in a fixed plane, e.g., the emitted light is also polarized, provided that the molecule is fixed in space. However, because the molecule is typically rotating and tumbling in space, the plane in which the fluoresced light is emitted varies with the rotation of the molecule. Restated, the emitted fluorescence is generally depolarized. The faster the molecule rotates in solution, the more depolarized it is. Conversely, the slower the molecule rotates in solution, the less depolarized, or the more polarized it is. The polarization value (P)

for a given molecule is proportional to the molecule's "rotational correlation time," (sometimes termed the "rotational relaxation time") or the amount of time it takes the molecule to rotate through an angle of 57.3° (1 radian). The smaller the rotational correlation time, the faster the molecule rotates, and the less polarization will be observed. The larger the rotational correlation time, the slower the molecule rotates, and the more polarization will be observed. Rotational correlation time is related to viscosity (η), absolute temperature (T), molar volume (V), and the gas constant (R). The rotational correlation time is generally calculated according to the following formula:

$$\text{Rotational Correlation Time} = 3\,\eta\,V/RT$$

As can be seen from the above equation, if temperature and viscosity are maintained constant, then the rotational correlation time, and therefore, the polarization value, is directly related to the molecular volume. Accordingly, the larger the molecule, the higher its fluorescent polarization value, and conversely, the smaller the molecule, the smaller its fluorescent polarization value.

In the performance of fluorescent binding assays, a typically small, fluorescently labeled molecule, e.g., a ligand, antigen, oligonucleotide or nucleic acid probe, etc., having a relatively fast rotational correlation time, is used to bind to a much larger molecule, e.g., a receptor protein, antibody, complementary nucleic acid target sequence etc., which has a much slower rotational correlation time, either on its own, or as a complex. The binding of the small labeled molecule to the larger molecule or in the larger complex significantly increases the rotational correlation time (decreases the amount of rotation) of the labeled species, namely the labeled complex over that of the free unbound labeled molecule. This has a corresponding effect on the level of polarization that is detectable. Specifically, the labeled complex presents much higher fluorescence polarization than the unbound, labeled molecule. The polarization value can then be used to determine the level of bound and free fluorescent compound.

Generally, the fluorescence polarization level is calculated using the following formula:

$$P=[I(\parallel)-I(\perp)]/[I(\parallel)+I(\perp)]$$

Where $I(\parallel)$ is the fluorescence detected in the plane parallel to the excitation light (also termed "polarized fluorescence"), and $I(\perp)$ is the fluorescence detected in the plane perpendicular to the excitation light (also termed "depolarized fluorescence"). Thus, as can be seen from this equation, the polarization value P is related to the ratio of polarized to depolarized fluorescence.

In performing screening assays, e.g., for potential inhibitors, enhancers, agonists or antagonists of the binding function in question, the change in fluorescence polarization of bound versus free labeled ligand is compared in the presence and absence of different compounds, to determine whether these different compounds have any effect on the binding function of interest. In particular, in the presence of inhibitors of the binding function, the fluorescence polarization will decrease, as more free, labeled ligand is present in the assay. Conversely, enhancers of the binding function will result in an increase in the fluorescent polarization, as more complex and less free labeled ligand are present in the assay.

III. Continuous Flow Assay Systems

It is an object of the present invention to provide methods, apparatuses and systems for performing fluorescent polarization assays in a high-throughput format. In particular, the present invention provides methods of performing continuous flow fluorescent polarization assays, and particularly, performing these assays in microfluidic systems. In general, continuous flow, high-throughput screening assays are described in commonly owned International Patent Application No. 98/00231, which is incorporated herein by reference in its entirety for all purposes.

In operation, a fluorescently labeled compound, e.g., a ligand, is flowed along a first microscale channel, e.g., a capillary tube or a channel in a microfluidic device. A second compound is also flowed along the first channel, the first and second compounds mixing in a first mixture. The first and second compounds interact within the microscale channel to produce a mixture that yields a fluorescent polarization level related to the level of free labeled compound relative to the level of bound labeled compound. In the flowing systems described herein, the mixture of the labeled ligand and unlabeled antiligand that is flowing through the channel produces a steady state signal corresponding to the level of bound complex. The steady state level of bound label is determined using the fluorescent polarization detection methods described herein, at a fixed point along the length of the first or analysis channel, and a steady state level of fluorescent polarization is observed.

In screening operations, test compounds are introduced into the main analysis channel and allowed to interact with the reaction mixture, e.g., the ligand and antiligand. In order to identify the point at which the test compound is introduced, these test compounds are typically introduced in plugs of fluid, e.g., discrete and relatively well defined plugs of fluid. Where a test compound affects the binding between the ligand and antiligand, it will produce a deviation in the amount of fluorescence polarization signal. For example, inhibitors of binding will result in a reduction in the amount of fluorescent polarization, while enhancers of binding will increase the amount of fluorescent polarization. Large numbers of different test compounds may be screened in this manner. Specifically, different compounds may be tested in a number of separate parallel channels, or they may be periodically introduced, serially, into the same channel or set of channels. This screening is then repeated with multiple different test compounds.

Test compounds are typically derived from large libraries of different compounds that may be naturally or synthetically derived. For example, compounds may be derived as extracts, or other purified, semipurified or nonpurified components of biological samples, e.g., plants, animals, fungi, insect, bacteria, etc. Typically, such test compounds are available in large libraries of different compounds that are synthetically produced, e.g., using combinatorial chemistry techniques. Alternatively and preferably, such libraries of test compounds are prepared by combinatorial chemical synthesis methods that produce large numbers of structurally and/or chemically diverse chemical compounds.

As noted above, the continuous flow format of the assay described herein, permits the screening of large numbers of different test compounds against a given binding function. Further, these screening assays are generally performed using extremely small volumes of reagents and test compounds, e.g., on the order of microliters of reagents for hundreds of thousands of screens. Further, because the fluorescent polarization assays are completely homogeneous, they are performed more rapidly than assays requiring additional steps, e.g., separation, solid phase immobilization, etc.

IV. Apparatus and Systems

A. System

The screening assays described above, are generally carried out in an appropriate assay system that permits analysis of large numbers of different test compounds, storage and analysis of data, and, optionally, computer assisted control and automation. A schematic illustration of an overall system is shown in Figure 1. As shown, the system 100

includes an analysis vessel, e.g., a microfluidic device 110. The system also includes a flow control system 120 operably coupled to the microfluidic device for directing flow of fluids through the channels of the microfluidic device 110. The flow control system 120 may employ a variety of different means for transporting fluid through channels in a controlled fashion, including through the application of pressure differentials (e.g., as shown), controlled electric fields, and the like. These systems are described in greater detail below. The system also typically includes a detector 130 for detecting the level of fluorescence polarization of material in the channels of the microfluidic device 110. A computer 140 is optionally provided for aiding in the functioning of the system 100. In particular, computer 140 is typically operably coupled to the detector 130 for receiving fluorescence polarization data, or even raw fluorescent intensity data from the detector, e.g., both parallel and perpendicular fluorescence. The computer is then programmed to analyze the data, store the data, and provide output in a user convenient format. The computer is optionally operably coupled to the flow control system 120. In this case, the computer is also appropriately programmed to instruct the flow controller in generating fluid flow through the channels of the microfluidic device 110 in a user selected profile. Also illustrated is a test compound/sample storage array 150, from which may be sampled large numbers of different test compounds for screening in accordance with certain aspects of the invention.

B. Detector

An example of a detection system is shown in Figure 2. As shown, the fluorescence polarization detector includes a light source 202 which generates light at an appropriate excitation wavelength for the fluorescent compounds that are present in the assay system. Typically, coherent light sources, such as lasers, laser diodes, and the like are preferred because of the highly polarized nature of the light produced thereby. The excitation light is directed through a polarizing filter 204, which passes only light in one plane, e.g., polarized light. The polarized excitation light is then directed through an optical train, e.g., including microscope objective 206, which focuses the polarized light onto the sample channel 208, through which the sample to be assayed is flowing, to excite the fluorescent label present in the sample.

Fluorescence emitted from the sample is then collected, e.g., through objective 206, and passed through the optical train including polarizing beam splitter 210, which separates the emitted fluorescence into a component that is in the plane parallel to the excitation light, and the plane perpendicular to the excitation light. The separated polarized light components are then separately directed to separate detectors 212 and 214, respectively,

where each component is quantified. Photomultiplier tubes (PMTs) are generally preferred as light detectors for the quantification of the light levels, but other light detectors are optionally used, such as photodiodes, CCDs, or the like.

The detector is typically coupled to a computer or other processor, which receives the data from the light detectors, and includes appropriate programming to compare the values from each detector to determine the amount of polarization from the sample, e.g., in accordance with the processes and equations set forth herein. The computer also typically displays the polarization data in an appropriate graphical format that is convenient for the user, e.g., as shown in Figure 6. In this particular example, the top line is parallel fluorescence data and the bottom line is perpendicular fluorescence data.

C. Microfluidic Device

As noted above, the fluorescent polarization assays described herein are preferably carried out in microfluidic devices and systems. As used herein, the term "microfluidic" generally refers to one or more fluid passages, chambers or conduits which have at least one internal cross-sectional dimension, e.g., depth, width, length, diameter, etc., that is less than 500μm, and typically between about 0.1 μm and about 500 μm. In the devices of the present invention, the microscale channels or chambers preferably have at least one cross-sectional dimension between about 0.1 μm and 200 μm, more preferably between about 0.1 μm and 100 μm, and often between about 0.1 μm and 20 μm. Accordingly, the microfluidic devices or systems prepared in accordance with the present invention typically include at least one microscale channel, usually at least two intersecting microscale channels, and often, three or more intersecting channels disposed within a single body structure. Channel intersections may exist in a number of formats, including cross intersections, "T" intersections, or any number of other structures whereby two channels are in fluid communication.

The body structure of the microfluidic devices described herein typically comprises an aggregation of two or more separate layers which when appropriately mated or joined together, form the microfluidic device of the invention, e.g., containing the channels and/or chambers described herein. Typically, the microfluidic devices described herein will comprise a top portion, a bottom portion, and an interior portion, wherein the interior portion substantially defines the channels and chambers of the device. In particular, these microfluidic devices are typically fabricated from two or more planar solid substrates. A series of interconnected grooves is generally fabricated into the surface of the first of the two substrates. The second substrate is then overlaid and bonded to the surface of the first substrate to seal the

grooves and define the integrated fluidic channels of the device. Typically one of the substrates includes one or more ports, e.g., holes, disposed through the substrate, that are positioned such that the holes are in fluid communication with the integrated channels in the complete assembled device. These holes then function as reservoirs for fluid introduction into the channels of the device, as well as providing electrical access points for the various channels for use in, e.g., controlled electrokinetic material transport systems.

One example of a microfluidic device useful in carrying out the assays described herein is illustrated in Figure 3. As shown, the device 300 includes a body 302 having a main analysis channel 304 disposed within its interior. At one terminus, main channel 304 is in fluid communication with waste reservoir 314. At the other terminus, main channel 304 is in fluid communication with an external sampling capillary or pipettor 320, via channel junction 316. Specifically, external capillary 320 is attached to the body 302 of the device 300, such that the channel within the capillary is fluidly connected to the main channel 304. Additional channels 326, 328, 330 and 332 intersect the main channel 304 and connect the main channel to reservoirs 306, 308, 310 and 312, respectively. These reservoirs 306-312 are used to introduce reagents into the main channel, including assay reagents, buffers, and the like. In operation, the assay reagents, e.g., ligand and antiligand, are flowed into the main channel 304 from, e.g., reservoirs 308 and 310. Appropriate diluents are also optionally added from reservoirs 306 and 312. As shown, fluid transport is driven by application of a vacuum source, e.g., to reservoir 314. Pressure or vacuum driven systems are described in greater detail below. Periodically, plugs of fluid containing test compounds are introduced into main channel 304 from capillary 320, e.g., by placing the open end of capillary 320 into a source of the test compound and drawing a volume of the test compound into the capillary channel and then up into the main channel 304.

The fluorescence polarization level of the assay mixture is then detected at a point in the main channel, e.g., detection window 334.

Another example of a microfluidic device for use in performing high-throughput screening assays using the fluorescence polarization assays described herein, is shown in Figure 4. As shown, the device 400 includes a body structure 402, which includes a main analysis channel 404 disposed within the body structure 402. At one terminus, the analysis channel is fluidly connected to waste well 406, while at the other terminus, the analysis channel is in fluid communication with the inlet 408 from an integrated external sample accessing capillary (not shown). In particularly preferred aspects, the external sample

accessing capillary is an electropipettor, e.g., as described in Published International Application No. 98/00707, incorporated herein by reference.

As shown, the device 400 also includes a fluorescently labeled ligand reservoir 410 that is in fluid communication with the main analysis channel 404, e.g., via fluorescent label channel 412, as well as a source of unlabeled antiligand 414, e.g., a receptor, antibody, etc., that is also fluidly connected to the main analysis channel, e.g., via antiligand channel 416. Buffer sources 418 and 424 are optionally provided in fluid communication with the fluorescent ligand channel and ligand complement channels, respectively, e.g., via channels 422 and 420, respectively. These buffer sources are used to control and vary the dilution of one or both of the fluorescent ligand and/or the ligand complement that are included in the assay system in main channel 404. As shown, the device also includes reservoirs 406a, 410a, 414a, 418a and 424a, which provide electrical access points to the reservoirs 406, 410, 414, 418 and 424, respectively, via an associated current passing channel 406b, 410b, 414b, 418b and 424b, respectively, which current passing channels allow for the passage of electrical current without allowing fluid flow, e.g., through the incorporation of salt bridges, or sufficiently shallow channels to prevent electroosmotic flow.

D. Material Transport System

As noted above, the direction and transport of fluids and other materials through the channels of the microfluidic devices as described herein, may be carried out using a number of different methods. For example, in some embodiments, pressure-based control of fluid movement is used. Such pressure-based control typically involves the application of pressure differentials across the length of channels through which fluid flow is desired. Application of pressure differentials may be accomplished through the incorporation of microfabricated pumps and valves within a microfluidic device. However, preferably, such pressure differentials are applied by applying a vacuum or pressure source to one or more termini of the channels of the microfluidic device. By regulating the applied pressures at the channel termini, one can effectively control fluid movement through those channels and through intersections of those channels with other channels. Optimally, a single vacuum source is operably coupled to a common reservoir, e.g., waste reservoir 314, as shown in Figure 3. As used herein, the term "operably coupled" refers to the connection between two elements, e.g., a vacuum source and a reservoir, so as to impart the functionality of one element to the other. In the case of vacuum sources, the operable coupling permits the transmission of the vacuum to the material in the reservoir. This creates a pressure differential along the length of each of the channels of the device which are generally open to

atmospheric pressure at other channel termini. Controlled differential flow through the various channels is then accomplished by configuring the channels to have appropriate pressure or flow resistances so as to yield a desired flow rate relative to the other channels. Such microfluidic channel networks incorporating these design characteristics are described in detail in U.S. Patent Application Nos. 09/238,467, filed January 28, 1999, which is incorporated herein by reference for all purposes.

In alternative aspects, the methods described herein are carried out using controlled electrokinetic material transport. As used herein, "electrokinetic material transport systems" include systems which transport and direct materials within an interconnected channel and/or chamber containing structure, through the application of electrical fields to the materials, thereby causing material movement through and among the channel and/or chambers, i.e., cations will move toward the negative electrode, while anions will move toward the positive electrode.

Such electrokinetic material transport and direction systems include those systems that rely upon the electrophoretic mobility of charged species within the electric field applied to the structure. Such systems are more particularly referred to as electrophoretic material transport systems. Other electrokinetic material direction and transport systems rely upon the electroosmotic flow of fluid and material within a channel or chamber structure which results from the application of an electric field across such structures. In brief, when a fluid is placed into a channel which has a surface bearing charged functional groups, e.g., hydroxyl groups in etched glass channels or glass microcapillaries, those groups can ionize. In the case of hydroxyl functional groups, this ionization, e.g., at neutral pH, results in the release of protons from the surface and into the fluid, creating a concentration of protons at near the fluid/surface interface, or a positively charged sheath surrounding the bulk fluid in the channel. Application of a voltage gradient across the length of the channel, will cause the proton sheath to move in the direction of the voltage drop, i.e., toward the negative electrode.

"Controlled electrokinetic material transport and direction," as used herein, refers to electrokinetic systems as described above, which employ active control of the voltages applied at multiple, i.e., more than two, electrodes. Rephrased, such controlled electrokinetic systems concomitantly regulate voltage gradients applied across at least two intersecting channels. Controlled electrokinetic material transport is described in Published PCT Application No. WO 96/04547, to Ramsey, which is incorporated herein by reference in its entirety for all purposes. In particular, the preferred microfluidic devices and systems described herein, include a body structure which includes at least two intersecting channels or

fluid conduits, e.g., interconnected, enclosed chambers, which channels include at least three unintersected termini. The intersection of two channels refers to a point at which two or more channels are in fluid communication with each other, and encompasses "T" intersections, cross intersections, "wagon wheel" intersections of multiple channels, or any other channel geometry where two or more channels are in such fluid communication. An unintersected terminus of a channel is a point at which a channel terminates not as a result of that channel's intersection with another channel, e.g., a "T" intersection. In preferred aspects, the devices will include at least three intersecting channels having at least four unintersected termini. In a basic cross channel structure, where a single horizontal channel is intersected and crossed by a single vertical channel, controlled electrokinetic material transport operates to controllably direct material flow through the intersection, by providing constraining flows from the other channels at the intersection. For example, assuming one was desirous of transporting a first material through the horizontal channel, e.g., from left to right, across the intersection with the vertical channel. Simple electrokinetic material flow of this material across the intersection could be accomplished by applying a voltage gradient across the length of the horizontal channel, i.e., applying a first voltage to the left terminus of this channel, and a second, lower voltage to the right terminus of this channel, or by allowing the right terminus to float (applying no voltage). However, this type of material flow through the intersection would result in a substantial amount of diffusion at the intersection, resulting from both the natural diffusive properties of the material being transported in the medium used, as well as convective effects at the intersection.

In controlled electrokinetic material transport, the material being transported across the intersection is constrained by low level flow from the side channels, e.g., the top and bottom channels. This is accomplished by applying a slight voltage gradient along the path of material flow, e.g., from the top or bottom termini of the vertical channel, toward the right terminus. The result is a "pinching" of the material flow at the intersection, which prevents the diffusion of the material into the vertical channel. The pinched volume of material at the intersection may then be injected into the vertical channel by applying a voltage gradient across the length of the vertical channel, i.e., from the top terminus to the bottom terminus. In order to avoid any bleeding over of material from the horizontal channel during this injection, a low level of flow is directed back into the side channels, resulting in a "pull back" of the material from the intersection.

One difficulty of electrokinetic injection is that, although small, the plugs are not ideal in shape. In particular, a plug produced by moving materials from one channel into

another by "pinched" injection has "wings" on the edges of the plug, giving it a wedge shape. Additional pinching of the plugs by flow of fluids or other materials from opposing channels simply flattens the wedge into a triangular shape. Typical methods do not produce a flat thin sample plug, which would be more ideal for ultrahigh-throughput sample manipulations (sample plugs could be stacked more closely in an analysis channel if their shapes were more regular, increasing the number of plugs that can be assayed, separated and/or flowed past a detection point).

To overcome this difficulty, sample materials are "pre-pinched" before being injected into an analysis channel. This pre-pinching procedure involves placing an intersection upstream of the analysis channel along a main flow channel. The upstream intersection has two opposing channels that simultaneously flow material into the main flow channel, causing lateral narrowing or "pinching" of sample material stream in the main flow channel. A plug of the pinched sample material stream is then injected into the analysis channel. Because of the pinching of the sample material in the main flow channel, the sample material injected into the analysis channel has a thinner and more regular shape. Alternatively, one can reverse the flow of the pinched material through the intersection for a very short period prior to injecting the material into the main analysis channel, all the while, maintaining the pinching flow from the side channels. Reversing the flow, e.g., by reversing the current is typically carried out for 1, 0.5, 0.1 seconds or less. This reversed flow yields a thinner band of material in the intersection which does not include the irregular shape of a typical pinched injection. Specifically, current that directs flow of material along a flow channel is briefly inverted as the flow of material passes an intersection that directs opposing lateral flow of material from side channels into the flow channel. This current inversion has the effect of backing up a portion of a material flow stream which has been "pinched" in the intersection by the opposing lateral flow of material, providing a flattened flow stream to the intersection. Sample plugs are flowed from the intersection into an analysis channel. Because the pinched flow stream has been backed into the intersection, the shape of plugs in the analysis channel is approximately as regular as if the material stream had been pre-pinched in the intersection. This is advantageous, e.g., because it reduces the complexity of the microscale system as compared to the pre-pinching approach, i.e., by eliminating the need for a pre-pinching intersection and associated channels and flow control elements.

In addition to pinched injection schemes, controlled electrokinetic material transport is readily utilized to create virtual valves which include no mechanical or moving parts. Specifically, with reference to the cross intersection described above, flow of material

from one channel segment to another, e.g., the left arm to the right arm of the horizontal channel, can be efficiently regulated, stopped and reinitiated, by a controlled flow from the vertical channel, e.g., from the bottom arm to the top arm of the vertical channel. Specifically, in the 'off' mode, the material is transported from the left arm, through the intersection and into the top arm by applying a voltage gradient across the left and top termini. A constraining flow is directed from the bottom arm to the top arm by applying a similar voltage gradient along this path (from the bottom terminus to the top terminus). Metered amounts of material are then dispensed from the left arm into the right arm of the horizontal channel by switching the applied voltage gradient from left to top, to left to right. The amount of time and the voltage gradient applied dictates the amount of material that will be dispensed in this manner.

Operable connection of electrical power supplies to the channels of the device is typically carried out via electrodes that are placed into contact with fluids in the reservoirs at the termini of the channels of the device, which electrodes are operably coupled to the power supply(ies). Examples of power supplies are described in commonly owned Published International Application No. 98/00705, which is incorporated herein by reference.

Although described for the purposes of illustration with respect to a four way, cross intersection, these controlled electrokinetic material transport systems can be readily adapted for more complex interconnected channel networks, e.g., arrays of interconnected parallel channels.

In operation, the fluorescent ligand and ligand complement are flowed into the main analysis channel 404, from sources 410 and 414, respectively. The mixture is then flowed along analysis channel 404 toward waste well 406, flowing past detection point 426. A fluorescent polarization detector, e.g., as described herein, is disposed adjacent to the detection point, and oriented to detect the level of fluorescent polarization within the analysis channel 404. Different test compounds are serially introduced into the main analysis channel 404 via an external sample accessing capillary and through inlet 408. As each test compound interacts with the mixture of fluorescent ligand and ligand binding complement, it may or may not have an effect on the relative level of binding between the components. Any changes in the relative level of binding are measured as a change in the relative level of fluorescence polarization at the detection point 426.

In still further optional embodiments, the systems used to practice the assay methods described herein operate in a mixed-mode method. Specifically, general fluid movement through the device is driven by pressure differentials, e.g., as described above.

However, electric fields are applied across a portion of the main analysis channel, in order to cause electrophoretic separation of reaction components prior to detection, e.g., products and reactants. Typically, separation is not necessary in systems employing fluorescence polarization. However, in the systems of the present invention, such separation allows one rapidly to determine the polarization values for completely bound and completely free fluorescent ligands, etc., by virtue of the rapid separations that are performable in these systems. In particular, the purified ligand-antiligand complex may be detected before significant amount of free ligand is generated as a result of the off-rate of the reactants. This is due, at least in part, to the continuing separation under an electric field, e.g., the leading edge of the complex species is free of unbound ligand. These polarization values are then useful as calibrators for subsequent polarization values where separation has not occurred, e.g., one knows the polarization values for free and bound, and can calculate the ratio of each from the polarization value of a mixture.

E. Computer Control and Data Analysis

As noted above, the systems of the present invention typically include a computer for use in storing and analyzing data received from the detectors, and/or for instructing the operation of the fluid flow controller.

In particular, the detector is typically coupled to a computer or other processor, which receives the data from the light detectors, and includes appropriate programming to compare the values from each detector to determine the amount of polarization from the sample. In particular, the computer typically includes software programming which receives as input the fluorescent intensities from each of the different detectors, e.g., for parallel and perpendicular fluorescence. The fluorescence intensity is then compared for each of the detectors to yield a fluorescence polarization value. One example of such a comparison is given by the equation:

$$P=[I(\parallel)-I(\perp)]/[I(\parallel)+I(\perp)]C$$

as shown above, except including a correction factor (C), which corrects for polarization bias of the detecting instrument. The computer determines the fluorescence polarization value for the reaction of interest. From that polarization value and based upon the polarization values for free and bound fluorescence, the computer calculates the ratio of bound to free fluorescence. Alternatively, the polarization values pre and post screening reaction are compared and a polarization difference (ΔP) is determined. The calculated polarization

differences may then be used as absolute values, e.g., to identify potential effectors of a particular reaction, or they may be compared to polarization differences obtained in the presence of known inhibitors or enhancers of the reaction of interest, in order to quantify the level of inhibition or enhancement of the reaction of interest by a particular compound.

In the case of high-throughput screening assay systems, the computer software optionally instructs the correlation of a particular screened result to a particular sample or sample acquisition location. This permits the investigator to identify the particular reagents employed in any one assay.

The computer system typically includes appropriate software for receiving user instructions, either in the form of user input into set parameter fields, e.g., in a GUI, or in the form of preprogrammed instructions, e.g., preprogrammed for a variety of different specific operations. The software then converts these instructions to appropriate language for instructing the operation of the material transport system, and/or for controlling, manipulating, storing etc., the data received from the detection system. In particular, the computer typically receives the data from the detector, interprets the data, and either provides it in one or more user understood or convenient formats, e.g., plots of raw data, calculated dose response curves, enzyme kinetics constants, and the like, or uses the data to initiate further controller instructions in accordance with the programming, e.g., controlling flow rates, applied temperatures, reagent concentrations, etc.

The present invention is further illustrated with reference to the following non-limiting examples.

EXAMPLES

The goal of the following experiments was to demonstrate the ability to perform binding assays in a continuous flow format using fluorescence polarization detection in a microfluidic system.

Fluorescence polarization was detected using an optical system generally as described above. In particular, fluorescent signals were measured in the epifluorescence mode using an inverted microscope (Nikon Eclipse TE300) equipped with two orthogonal photomultiplier tubes (PTI D-104 Microscope Photometer). The excitation light source used was a tunable Argon ion laser (457-514 nm). A dichroic filter (High Q FITC Filter Set#41001, Chroma Technology Corp.) was used for selecting the excitation and emission wavelengths for the fluorescein labeled peptide. For polarization measurements, an Oriel VIS-NIR dichroic sheet polarizer (Model #27345, wavelength range 380-770 nm) mounted

on a 2 inch rotator was placed in the laser beam path before the microscope optics. The emitted fluorescence was passed through a Newport broadband polarizing cube beamsplitter (#05FC16PB.3, 12.7 mm) and the parallel and perpendicular beams were then detected by the two orthogonal PMTs.

Fluorescence polarization was calculated according to the formula:

$$P = \frac{[I(\parallel) - GI(\perp)]}{[I(\parallel) + GI(\perp)]}$$

where G is a correction factor resulting from the instrumentation and electronics of the detector used. Typically, in standard fluorescent polarization detection, the G factor is determined by exciting the sample in the horizontal plane and detecting fluorescence (Ih) in both the horizontal (Ih_h) and vertical planes (Ih_v) and comparing the two, the ratio of vertical to horizontal fluorescence providing the correction factor ($G=Ih_v/Ih_h$). In the case of microfluidic systems, however, the correction factor G is determined by measuring a sample of known fluorescence polarization in the system to be used in the assay, and correlating the known polarization value with the determined polarization value. The polarization values were calculated in an Excel™ spreadsheet in which parallel and perpendicular fluorescent intensities were corrected for background by subtracting the parallel and perpendicular intensities for the buffer, and inserting the correction factor (G).

In the experiments, the model binding system included a phosphoprotein receptor and a fluorescently labeled peptide ligand that is known to bind the phosphoprotein receptor. Unlabeled peptide ligand was used as a model competitive inhibitor to demonstrate changes in fluorescent polarization resulting from that inhibition. The various assay components were all in 50 mM HEPES buffer, 0.05 % Tween-20, 5% glycerol, 10 mM DTT, 500 mM betaine, at pH 7.4.

A microfluidic device having the channel and well geometry shown in Figure 5 was used to carry out the model assay, with the different assay components and buffers placed into the reservoirs as shown. Controlled electrokinetic transport was used to move the various assay components into the main analysis channel and vary their concentrations.

Initially, 46 nM of the fluorescently labeled peptide was flowed along the main channel concurrently with the phosphoprotein which was toggled between two concentrations of 461 nM and 154 nM. The fluorescent intensity data for this continuous flow assay, as measured using fluorescence polarization detection is shown in Figure 6. As

shown in Figure 7, the fluorescent polarization level went from 0.08 for the free fluorescent peptide, to 0.108 for peptide and 154 nM phosphoprotein receptor, to 0.128 for peptide ligand and 461 nM phosphoprotein receptor. This data illustrates the increasing level of polarization with increasing fraction of bound labeled peptide ligand. The polarization levels are graphically illustrated in Figure 7, as a function of receptor, e.g., phosphoprotein receptor, concentration.

Figure 8 shows fluorescent intensity data for fluorescence polarization detection of binding between the labeled peptide ligand (33 nM) and the phosphoprotein receptor (333 nM)(middle peak). The two different plot lines represent the fluorescence in each of the parallel (top line) and perpendicular (bottom line) planes to the excitation light. The difference between the lines is a measure of the level of polarization. As can be seen from the middle peak in Figure 8, significant polarization is observed upon binding of the phosphoprotein receptor to the labeled peptide ligand. Further, differential polarization levels were observed in the presence of three different concentrations of a competitor (1.6 nM, 0.8 nM and 0.27 nM unlabeled peptide ligand). Figure 9, shows a plot of the level of polarization versus the concentration of competitor (unlabeled peptide ligand), clearly demonstrating the competitive inhibition of binding by the unlabeled peptide ligand, and its detection by fluorescent polarization.

Additional experiments were performed demonstrating the efficacy of the methods described herein. In these additional experiments, a microfluidic device having the layout shown in Figure 10 was used. This device is identical to that shown in Figure 5, except the reagent placement in the reservoirs is changed as indicated. Reagents are manually pipetted into the wells connected by microchannels. Reagents are electrokinetically pumped through the chip by appropriate switching of electric fields between reservoirs. Reaction times are determined by the flow rate and channel dimensions of the chip. On-chip dilution of reagents is carried out by varying the ratio of current applied to each reagent supply channel and its paired buffer channel.

Figure 11 is an example of fluorescence polarization data carried out in an inverted fluorescence microscope. Fluorescence intensities were measured in parallel (top line) and perpendicular (bottom line) polarization planes by exciting with polarized light and monitoring the emitted fluorescence using a polarizing beam-splitter cube and dual photomultipliers, as described above. The microfluidic device was programmed to cycle between states consisting of: (1) buffer only, (2) labeled peptide plus target protein, or (3)

labeled peptide plus target protein in the presence of different concentrations of the unlabeled peptide.

Figure 12 shows a saturation binding curve for the N1 target protein titrated into a fixed concentration (25 nM) of the labeled probe peptide. Nonlinear least squares fitting to a binding equation that corrects for ligand depletion yields an estimate of 112.7 nM for the K_d of the binding interaction.

Figure 13 is a competition binding curves for the N1 target protein (350 nM) using 25 nM of a known competitor (NL932) and an irrelevant compound (N16914). The reaction time is 70 seconds.

Figure 14 is a competition binding curve for the N1 target protein (400 nM) using 25 nM of a known competitor (NL932). Nonlinear least squares fitting of the data to a one-site competitive binding model and correction for ligand depletion yields an estimate of K_i - 279 nM.

A fluorescence polarization experiment was also run using a microfluidic device that included an external sampling capillary or pipettor integrated with the channel network of the device. The channel layout of the device is shown in Figure 15. Briefly, the external pipettor element (not shown) interfaces with the main reaction channel 1504 at the junction point 1506. Fluid movement through the channels of the device, as well as reagent introduction through the pipettor element was pressure controlled by applying a vacuum to the waste well 1508. Additional reagents for the analysis were introduced from well 1510 and 1514 via channels 1512 and 1516, respectively. The relative fluorescence polarization of the assay mixture was detected at the detection point 1518 using a fluorescence polarization detection scheme as described above.

The reagents used for the assay were CFR-binding protein (receptor) and Fluo-sauvagine (labeled peptide) which binds the CFR binding protein. Unlabeled Sauvagine was used as a model inhibitor. Experiments were conducted in 10 mM PBS, 0.02% NP-40, pH 7.4. Unless otherwise mentioned, all concentrations reported below are final concentrations after being corrected for dilution factors. In all the experiments reported below, the detection point was positioned such that it took 30 seconds for a dye to reach the detection point.

Figure 16 represents a plot of the polarization vs. time of a binding experiment where the labeled peptide is constantly flowing from well 1510, through channel 1512 and into channel 1504 and the external pipettor element is alternating between microtiter plate wells to draw in alternating fluid plugs containing the binding protein (90 nM well

concentration) and buffer. From the data in Figure 16, it is clearly evident that the polarization remains the same when just the labeled peptide is flowing (Pc = 0.056) and goes up (to Pc = 0.105) when the protein that is being sipped up from the microtiter plate well mixes with the peptide. These values are in excellent agreement with polarization values obtained both from spectrofluorimeter and premixed experiments indicating that under the particular conditions of the above experiment, equilibrium has been reached.

Figure 17 represents a plot of a competitive inhibiting binding assay using fluorescence polarization detection where the binding protein (90 nM) (in well 1510) and the labeled peptide (50 nM) (in well 1514) are continually flowing while different concentrations of competitor are being sipped up from the microtiter plate, each concentration of inhibitor being alternated by buffer wells. The concentrations of inhibitor used were 50 nM, 200 nM, 400 nM, 800 nM, and 1000 nM. It is clearly apparent that increasing concentrations of inhibitor, as expected, causes successively increasing drops in polarization.

Figure 18 is the raw intensity data for the parallel (top line) and perpendicular (bottom line) components described in Figure 15. The polarization plots were obtained using software developed internally.

All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference. Although the present invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the invention.

What is claimed is:

1. A method of detecting an interaction of at least first and second compounds, comprising:

flowing the first compound along a first microscale channel;

flowing the second compound along the first microscale channel whereby the first and second compounds mix in a first mixture, wherein at least one of the first and second compounds has a fluorescent label associated therewith;

detecting a level of polarized fluorescence emitted from the fluorescent label in the first mixture; and

determining an amount of interaction between the first and second compounds from the level of polarized fluorescence emitted from the first mixture.

2. The method of claim 1, wherein the first and second compounds comprise first and second members of a specific binding pair, respectively.

3. The method of claim 2, wherein the first compound comprises a receptor molecule and the second compound comprises a ligand for the receptor molecule.

4. The method of claim 2, wherein the first compound comprises an antibody and the second compound comprises an antigen or antibody binding epitope.

5. The method of claim 2, wherein the first and second compounds comprise complementary nucleic acid sequences.

6. The method of claim 5, wherein the complementary nucleic acids are independently selected from ribonucleic acids, deoxyribonucleic acids, and peptide nucleic acids.

7. The method of claim 1, further comprising flowing a third compound into the microscale channel whereby the third compound mixes with the first and second compounds in the first mixture, and wherein the determining step comprises determining the level of interaction of the first and second compounds in the presence of the third compound.

8. The method of claim 7, wherein the third compound is introduced into the microscale channel in a discrete fluid plug.

9. The method of claim 8, further comprising flowing a fourth compound into the microscale channel as a discrete fluid plug, the fourth compound mixing with the first and second compounds in the first mixture, and wherein the determining step comprises determining the level of interaction of the first and second compounds in the presence of the fourth compound.

10. The method of claim 1, wherein the detecting step comprises directing a polarized excitation light at the first mixture in the first microscale channel, and detecting a level of emitted fluorescence in planes parallel and perpendicular to a plane of the polarized excitation light.

11. The method of claim 1, wherein in the determining step, the interaction of the first and second compounds comprises a binding of the first compound to the second compound.

12. An assay system, comprising:

a microfluidic device comprising a body having at least a first microscale channel disposed therein;

a material transport system for transporting first and second compounds along the first channel in a first mixture; and

a fluorescence polarization detector in sensory communication with the first microscale channel.

13. The assay system of claim 12, wherein the microfluidic device comprises at least a second microscale channel portion disposed within the body that intersects and is in fluid communication with the first microscale channel, the second microscale channel portion being fluidly connected to a source of the first compound.

14. The assay system of claim 13, wherein the microfluidic device comprises at least a third microscale channel portion disposed within the body that intersects

and is in fluid communication with the first microscale channel, the third channel portion being fluidly connected to a source of the second compound.

15. The assay system of claim 14, wherein the microfluidic device comprises at least a fourth microscale channel portion that intersects and is in fluid communication with the first microscale channel, the fourth channel portion being fluidly connectable to at least a source of the third compound.

16. The assay system of claim 15, wherein the fourth channel portion comprises a capillary channel disposed in a capillary element attached to the body at a first end and being open at a second end, the second end that is connectable to at least a source of a third compound.

17. The assay system of claim 12, wherein the material transport system comprises a pressure or vacuum source operably coupled to the first microscale channel to impart a pressure differential along a length of the first channel.

18. The assay system of claim 12, wherein the material transport system comprises an electrokinetic material transport system.

19. The assay system of claim 18, wherein the electrokinetic material transport system comprises at least first and second electrodes disposed in reservoirs at opposite ends of the first channel, each of the electrodes being electrically coupled to an electrical power supply.

20. The assay system of claim 12, wherein the material transport system comprises:

a pressure or vacuum source operably coupled to the first microscale channel to impart a pressure differential across a length of the first microscale channel; and

at least two electrodes operably coupled to the first microscale channel to impart an electric field across a portion of the first microscale channel.

21. The assay system of claim 12, wherein the fluorescent polarization detector comprises:

a light source for directing a polarized excitation light at the first microscale channel;

an optical train for receiving emitted fluorescence from the first mixture in the first microscale channel, and for separating fluorescence that is parallel to a plane of the excitation light from fluorescence that is perpendicular to the plane of the excitation light; and

first and second light detectors for separately detecting the fluorescence that is parallel to the plane of the excitation light, and the fluorescence that is perpendicular to the plane of the excitation light, respectively.

22. The assay system of claim 21, further comprising a computer connected to the first and second light detectors, the computer being programmed to calculate a fluorescent polarization value of the first mixture from the detected fluorescence that is parallel to the plane of the excitation light and the detected fluorescence that is perpendicular to the plane of the excitation light.

23. A method of screening a test compound for an effect on an interaction of at least first and second compounds, comprising:

flowing a first mixture comprising first and second interacting compounds along a first microscale channel, in the presence of the test compound, at least one of the first and second compounds having a fluorescent label associated therewith;

detecting a level of polarized fluorescence emitted from the fluorescent label in the first mixture in the presence of the test compound; and

comparing the level of fluorescent polarization emitted from the fluorescent label in the first mixture in the presence of the test compound to a level of fluorescent polarization of the fluorescent label in the first mixture in an absence of the test compound.

24. A method of assaying an association of a first compound with a second compound, comprising:

flowing a fluid comprising the first and second compounds through a first microscale channel, at least one of the first and second compounds having a fluorescent label associated therewith;

exciting the fluorescent label in the first channel with a polarized excitation light; and

detecting polarized and depolarized fluorescence from the fluorescent label, an amount of polarized fluorescence relative to depolarized fluorescence being indicative of a level of interaction between the first and second compounds.

25. The use of fluorescence polarization to detect an interaction between first and second compounds flowing through a microscale channel.

26. The use of fluorescence polarization to detect an effect of a test compound on an interaction between first and second compounds flowing through a microscale channel.

27. The use of either of claims 25 or 26, wherein at least one of the first and second compounds has a fluorescent label associated therewith.

28. The use of either of claims 25 or 26, wherein the microscale channel is disposed in a microfluidic device.

29. The use of either of claims 25 or 26, wherein the first and second compounds comprise members of a specifically associating binding pair.

30. The use of claim 29, wherein the first and second compounds comprise a ligand and a receptor.

31. The use of claim 29, wherein the first and second compounds comprise an antibody and an antigen.

32. The use of claim 29, wherein the first and second compounds comprise complementary nucleic acid sequences.

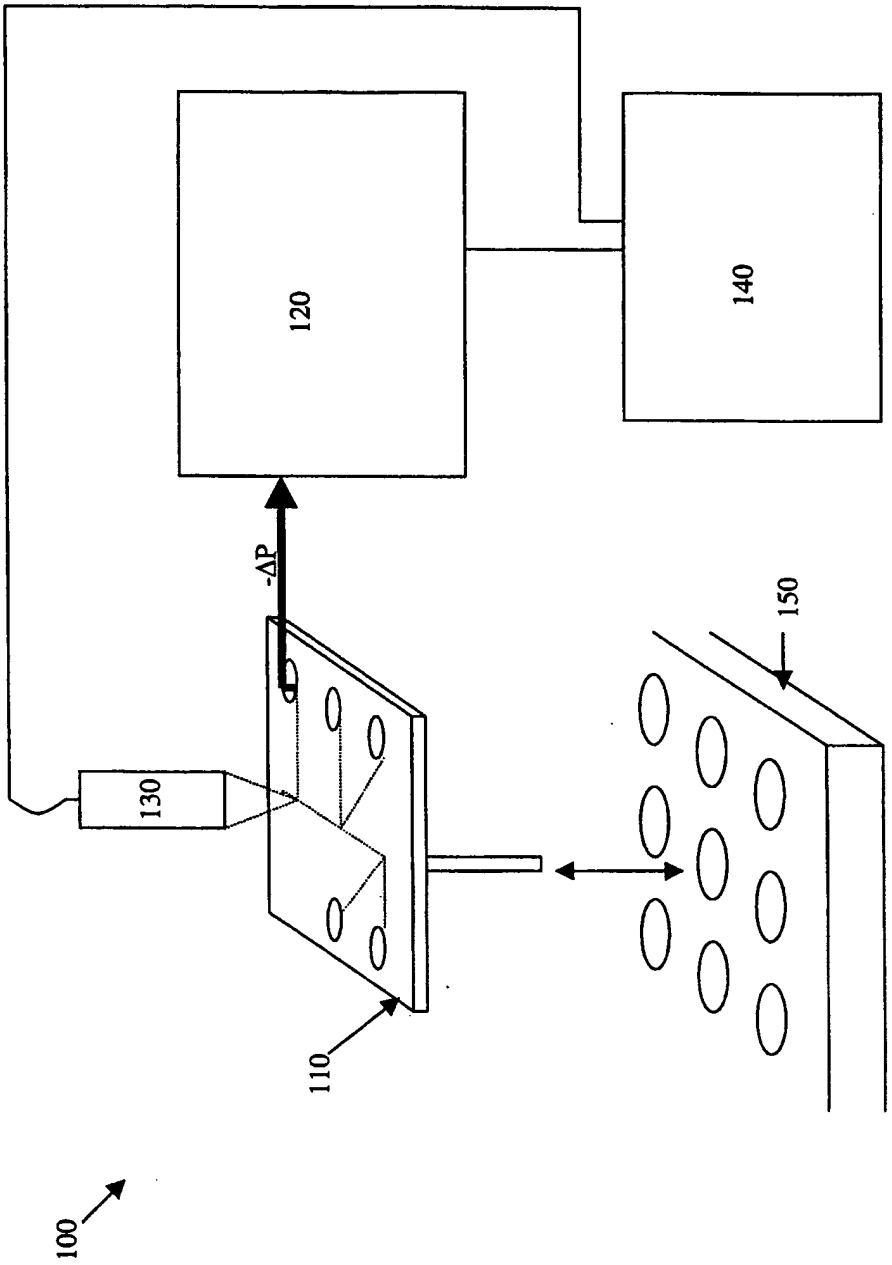


Figure 1

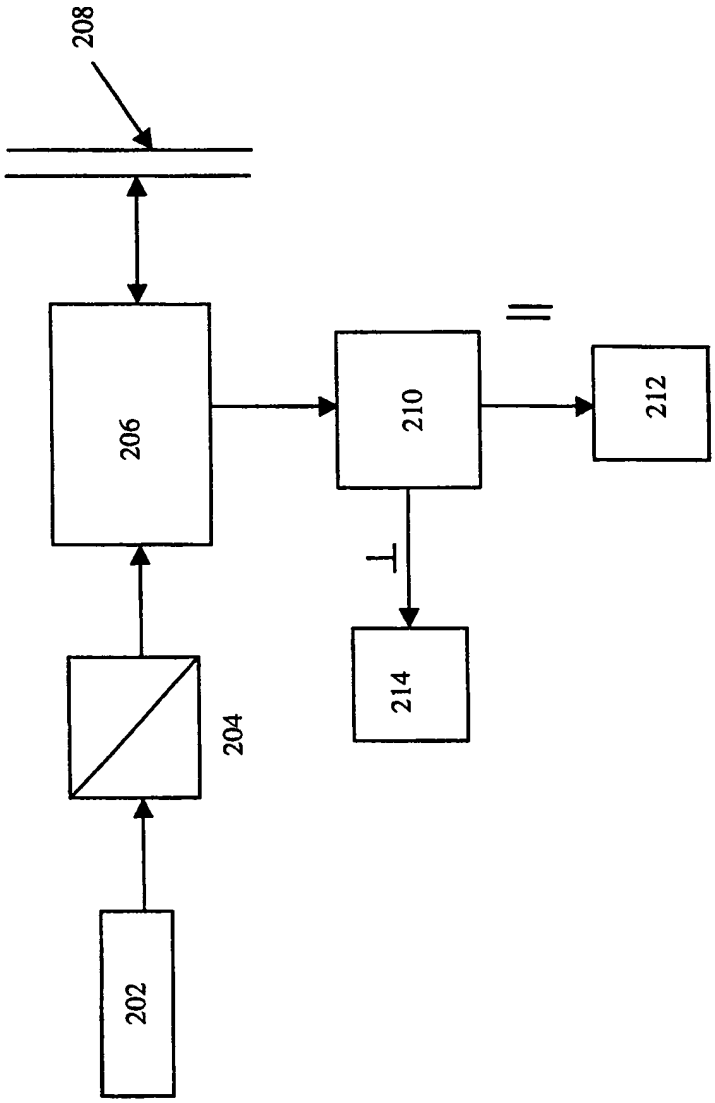


Figure 2

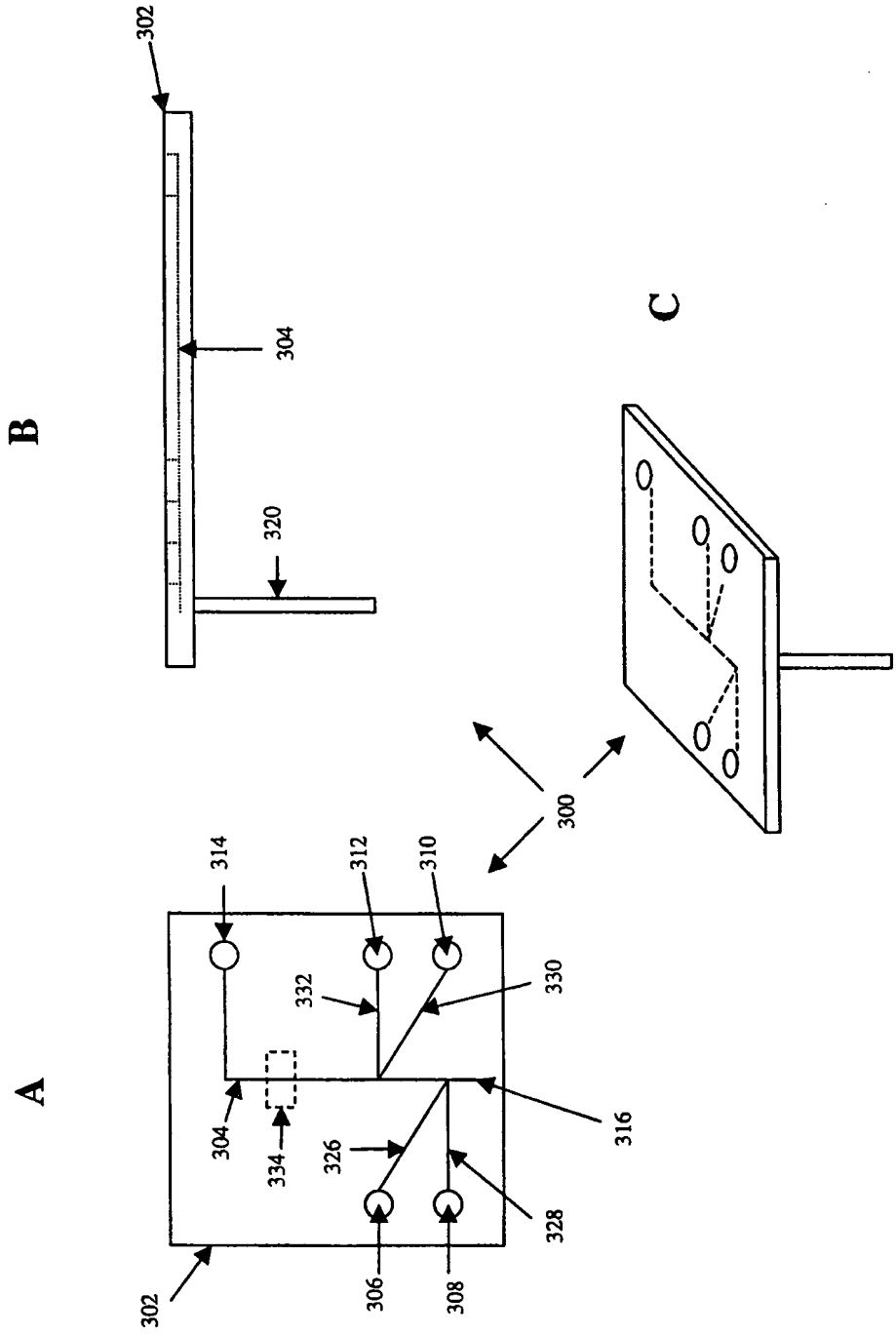
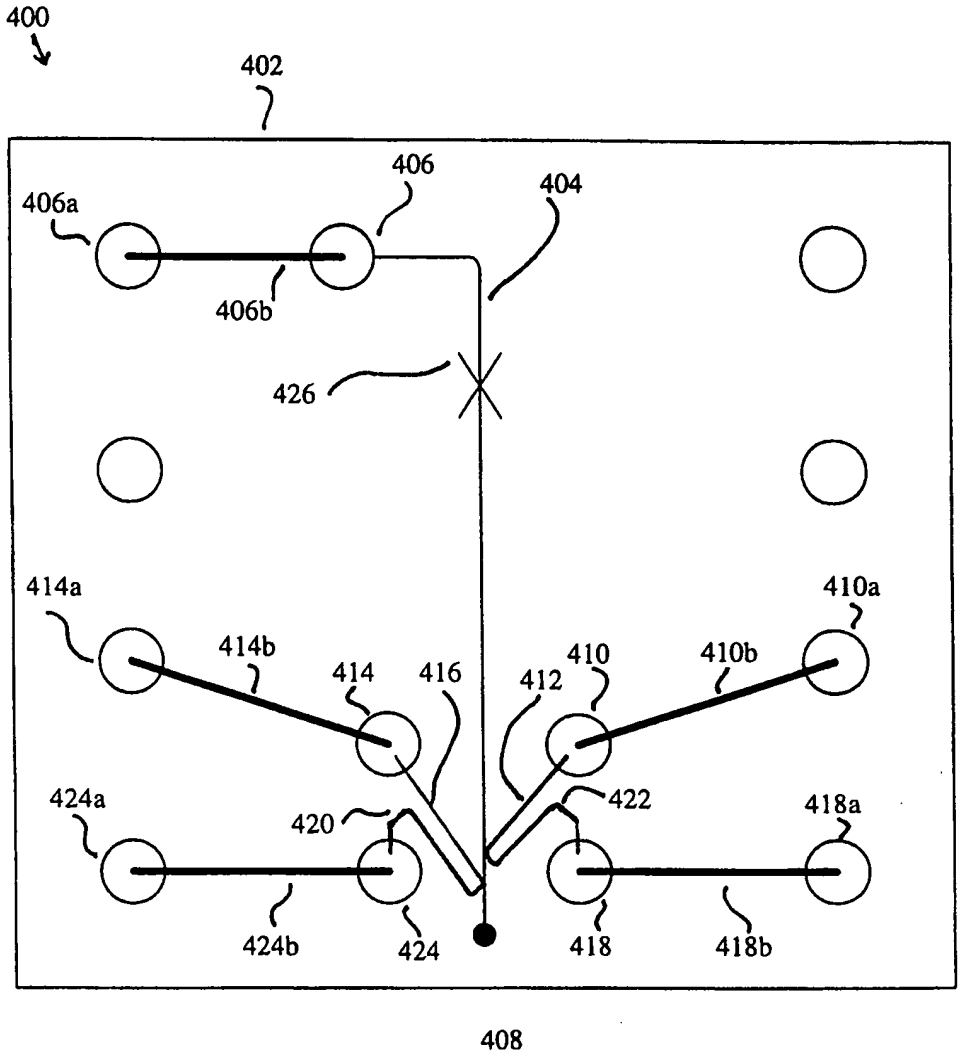


Figure 3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418b
408

Figure 4

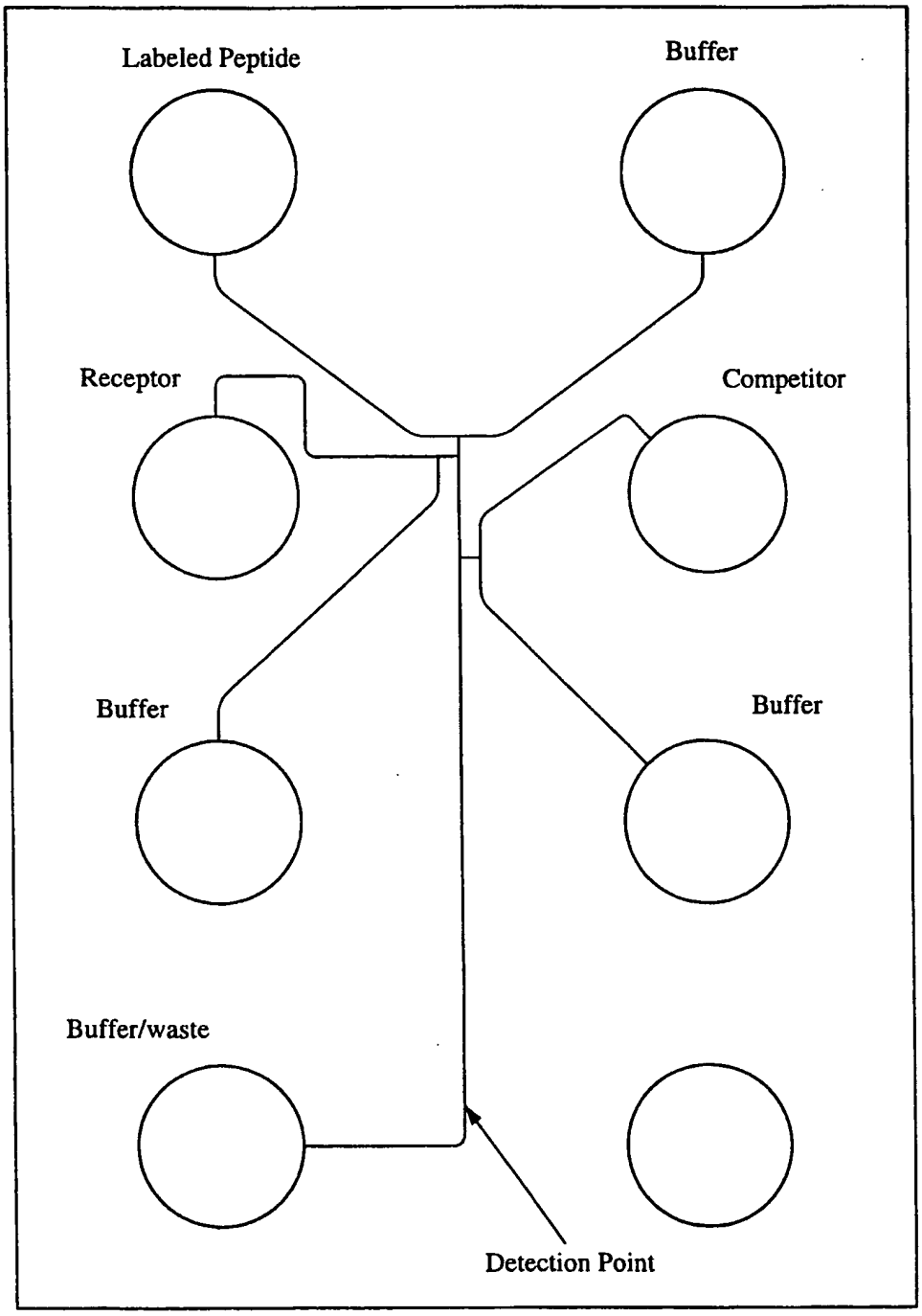
Labeled Peptide
Buffer
Receptor
Competitor
Buffer
Buffer
Buffer/waste
Detection Point

Figure 5

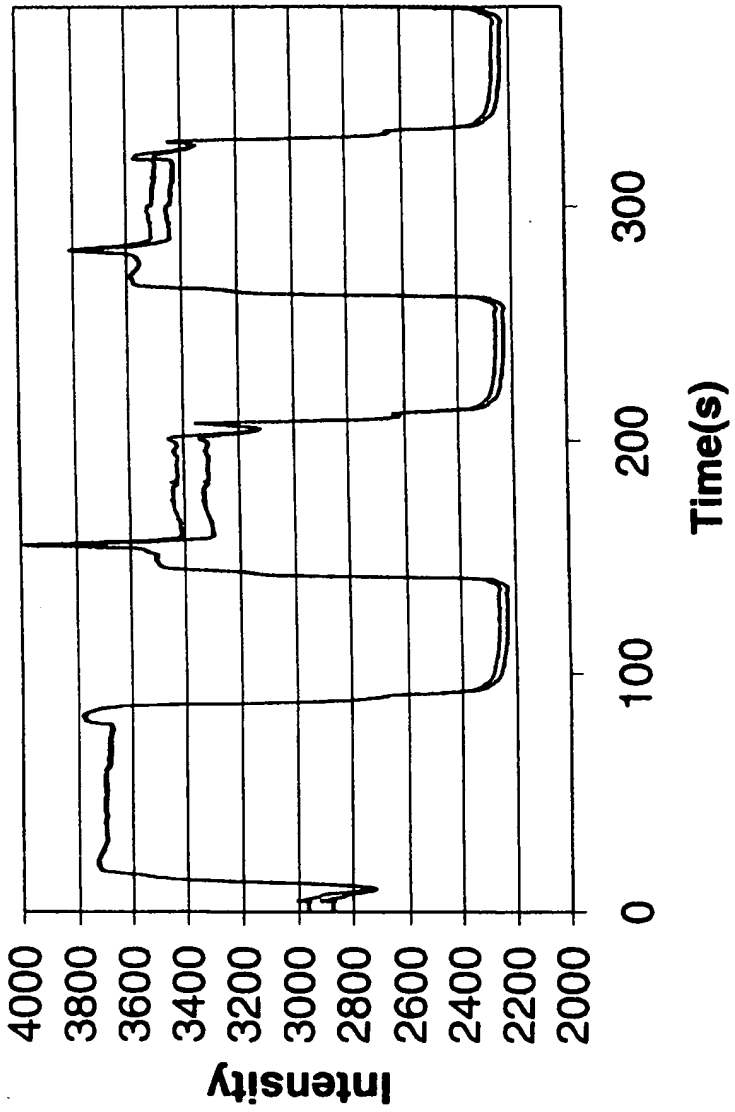
Intensity
4000
3800
3600
3400
3200
3000
2800
2600
2400
2200
2000
0
100
200
300
Time(s)

Figure 6

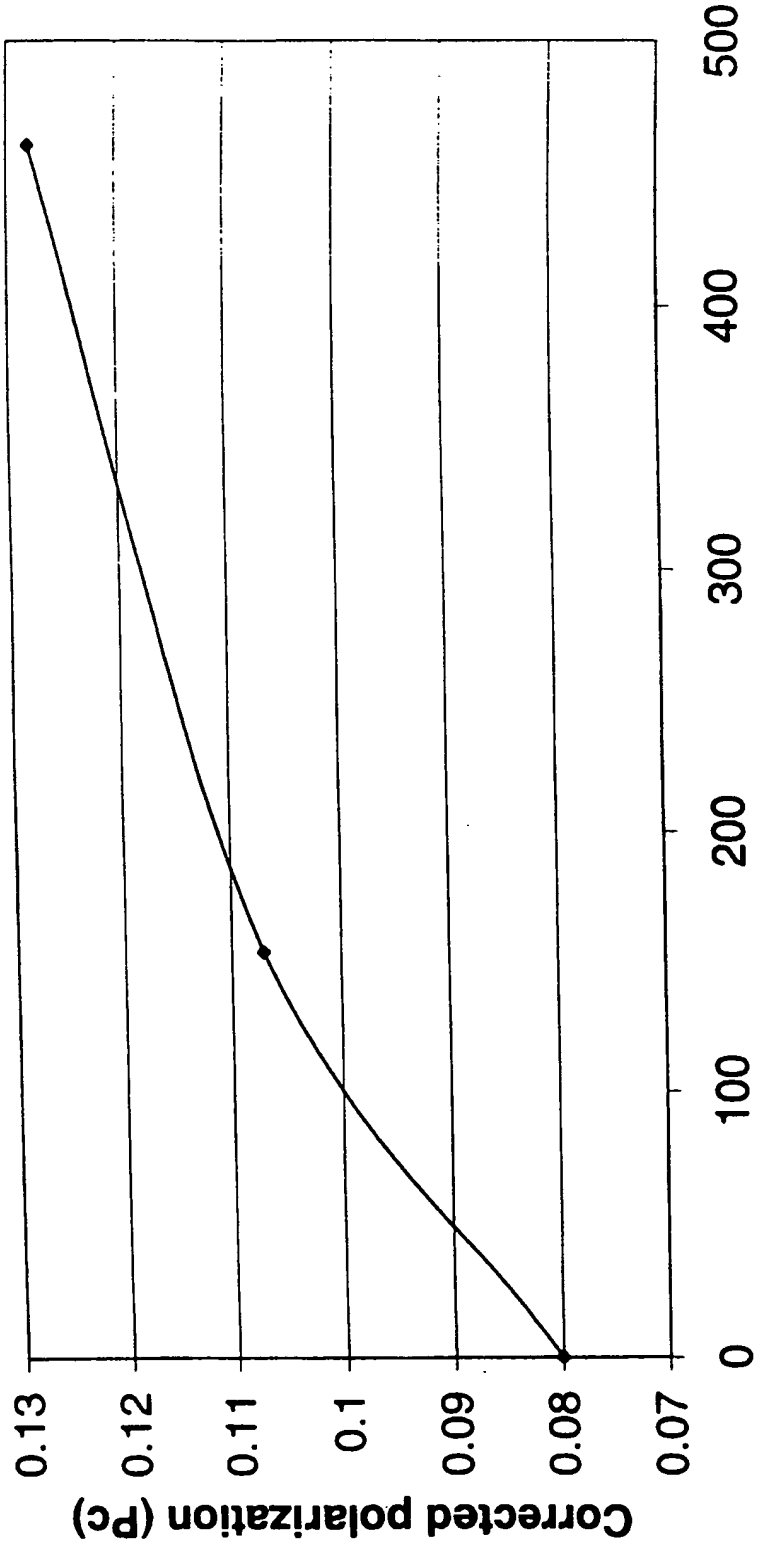
Corrected polarization (Pc)
0.13
0.12
0.11
0.1
0.09
0.08
0.07
0
100
200
300
400
500
[Phosphoprotein], nM

Figure 7

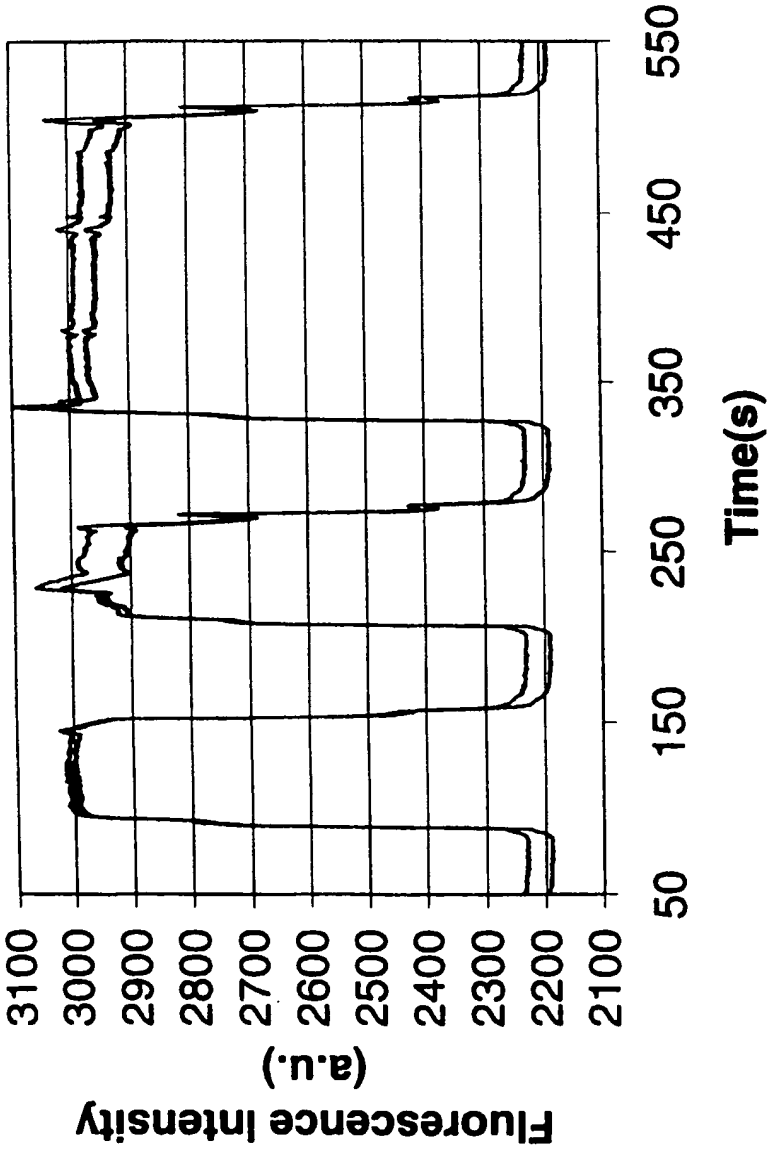
Fluorescence Intensity
(a.u.)
3100
3000
2900
2800
2700
2600
2500
2400
2300
2200
2100
50
150
250
350
450
550
Time(s)

Figure 8

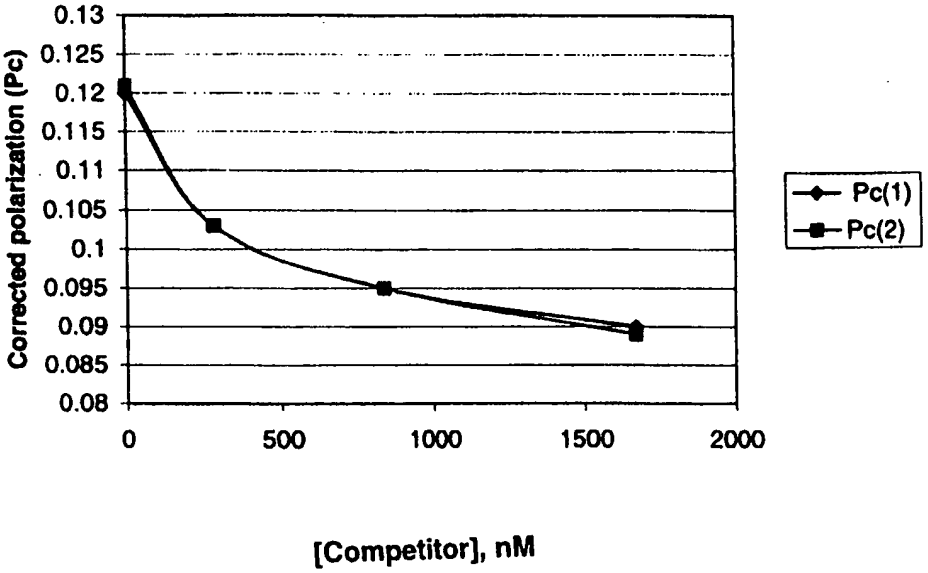
0.13
0.125
0.12
0.115
0.11
0.105
0.1
0.095
0.09
0.085
0.08
Corrected polarization (Pc)
0
500
1000
1500
2000
[Competitor], nM
Pc(1)
Pc(2)

Figure 9

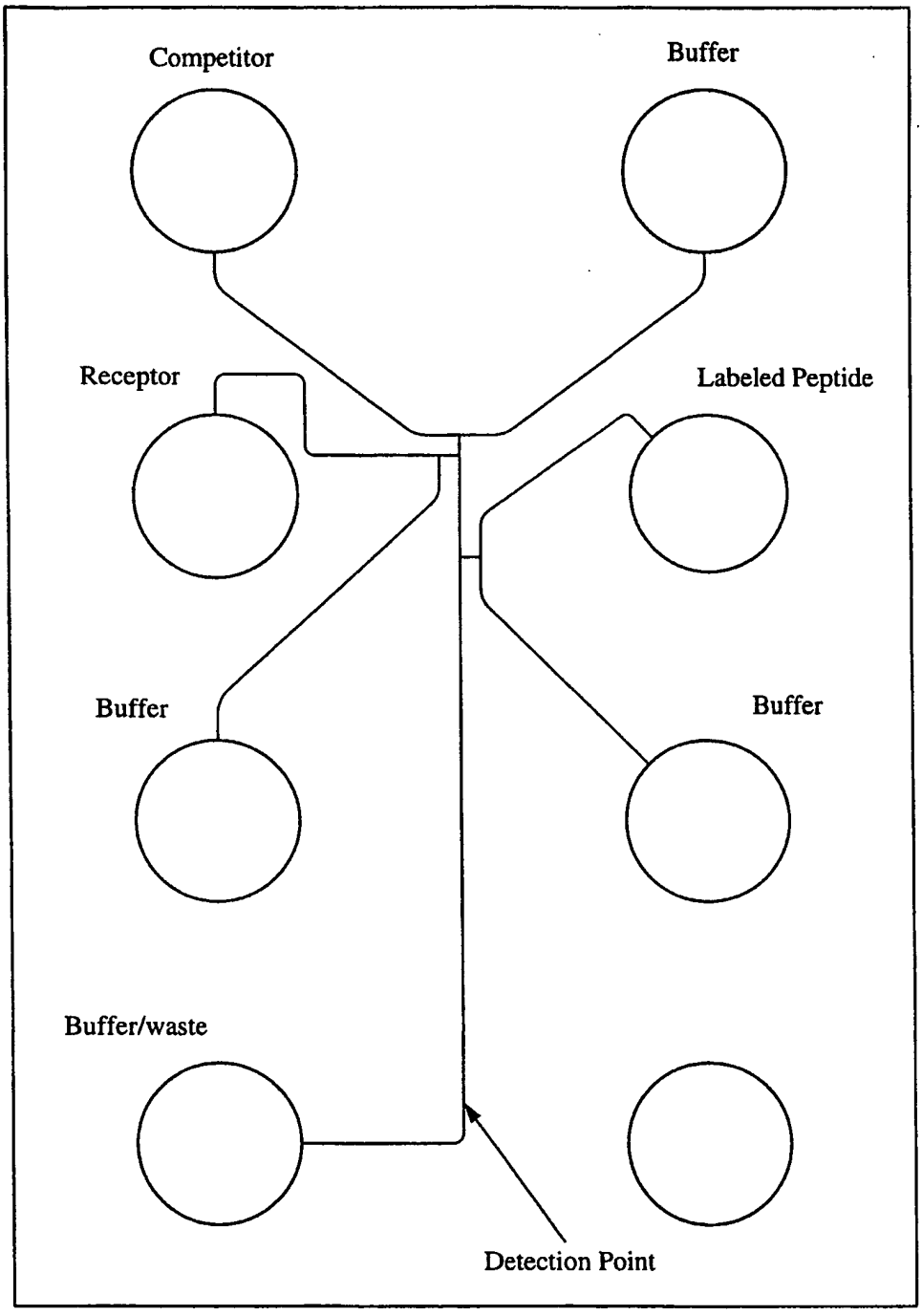
Competitor
Buffer
Receptor
Labeled Peptide
Buffer
Buffer
Buffer/waste
Detection Point

Figure 10

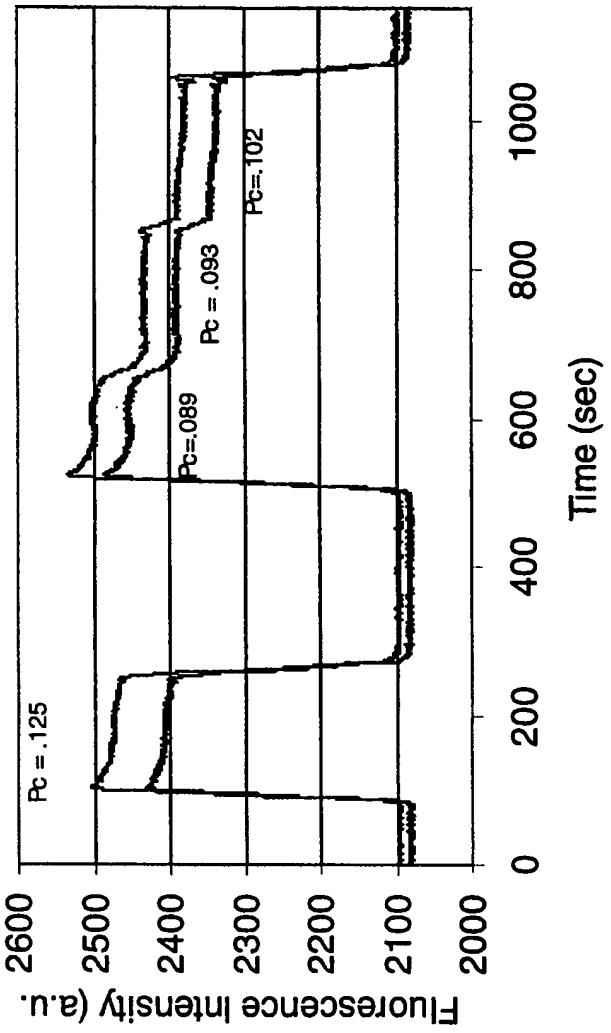
Fluorescence Intensity (a.u.
2600
2500
2400
2300
2200
2100
2000
0
200
400
600
800
1000
Time (sec)
Pc = .125
Pc=.089
Pc = .093
Pc=.102

Figure 11

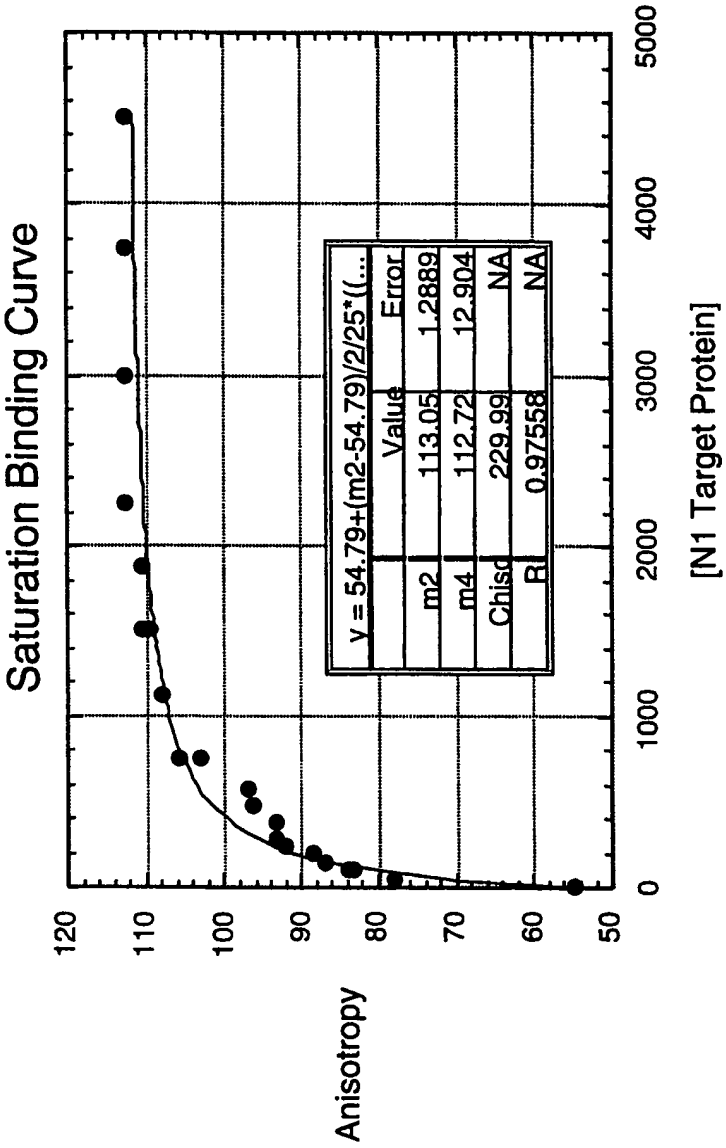
Saturation Binding Curve
y = 54.79+(m2-54.79)/2/25*((...
Value Error
m2 113.05 1.2889
m4 112.72 12.904
Chisq 229.99 NA
R 0.97558 NA
Anisotropy
120
110
100
90
80
70
60
50
0
1000
2000
3000
4000
5000
[N1 Target Protein]

Figure 12

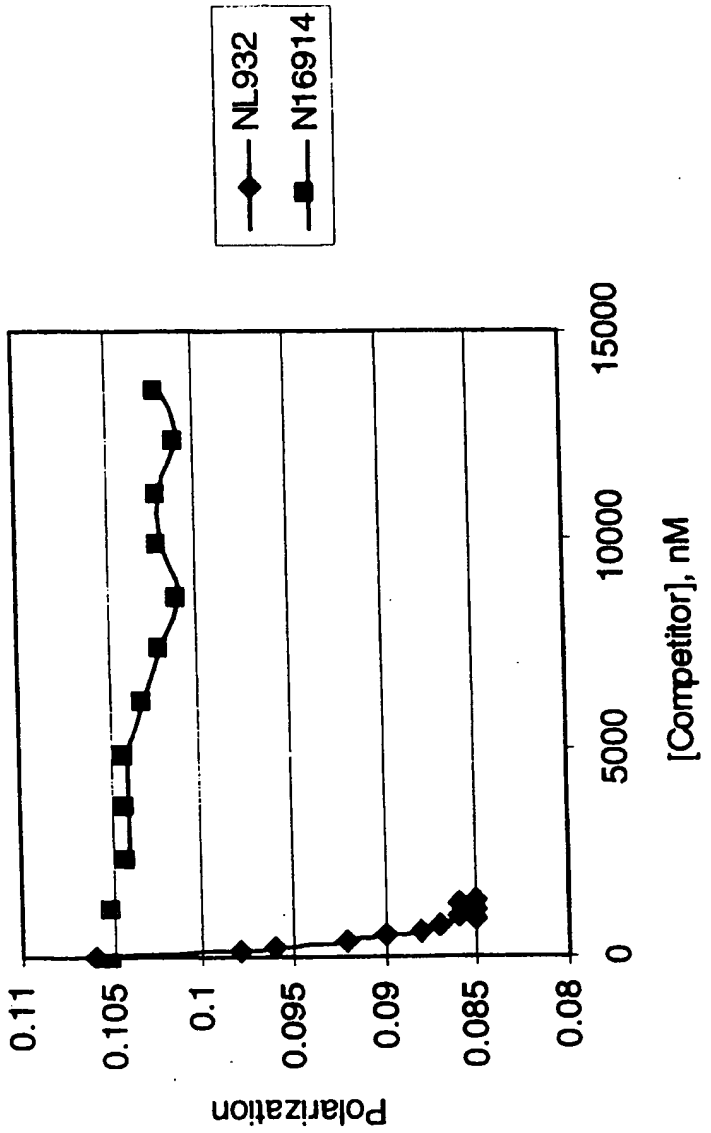
NL932
N16914
Polarization
0.11
0.105
0.1
0.095
0.09
0.085
0.08
0
5000
10000
15000
[Competitor], nM

Figure 13

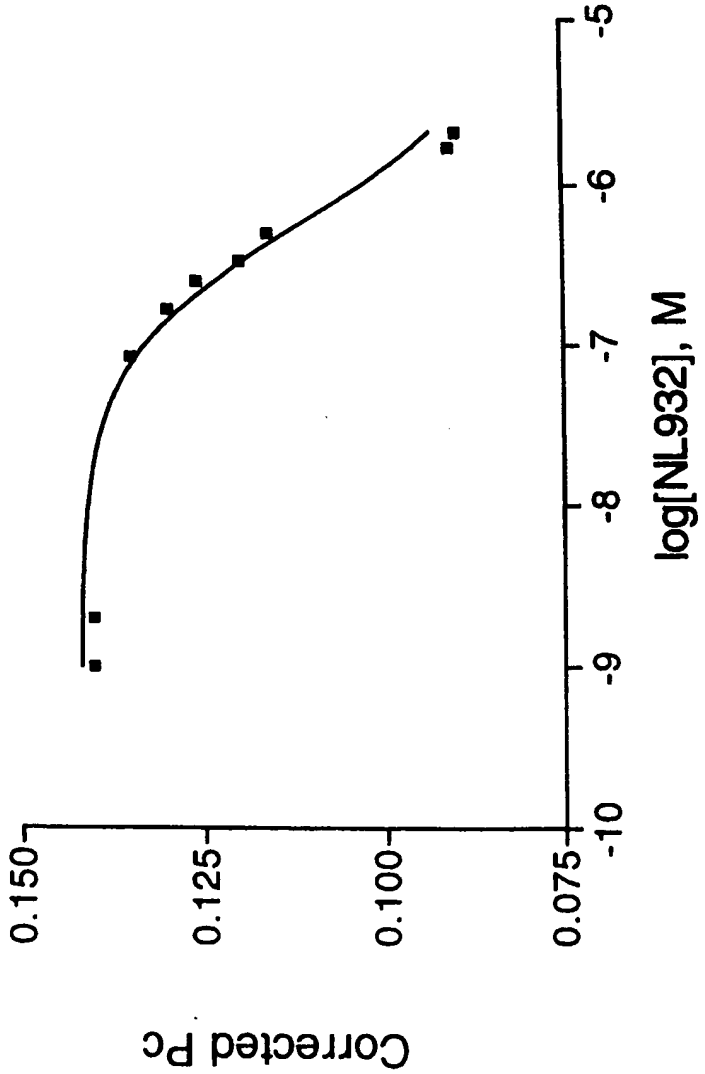

Figure 14

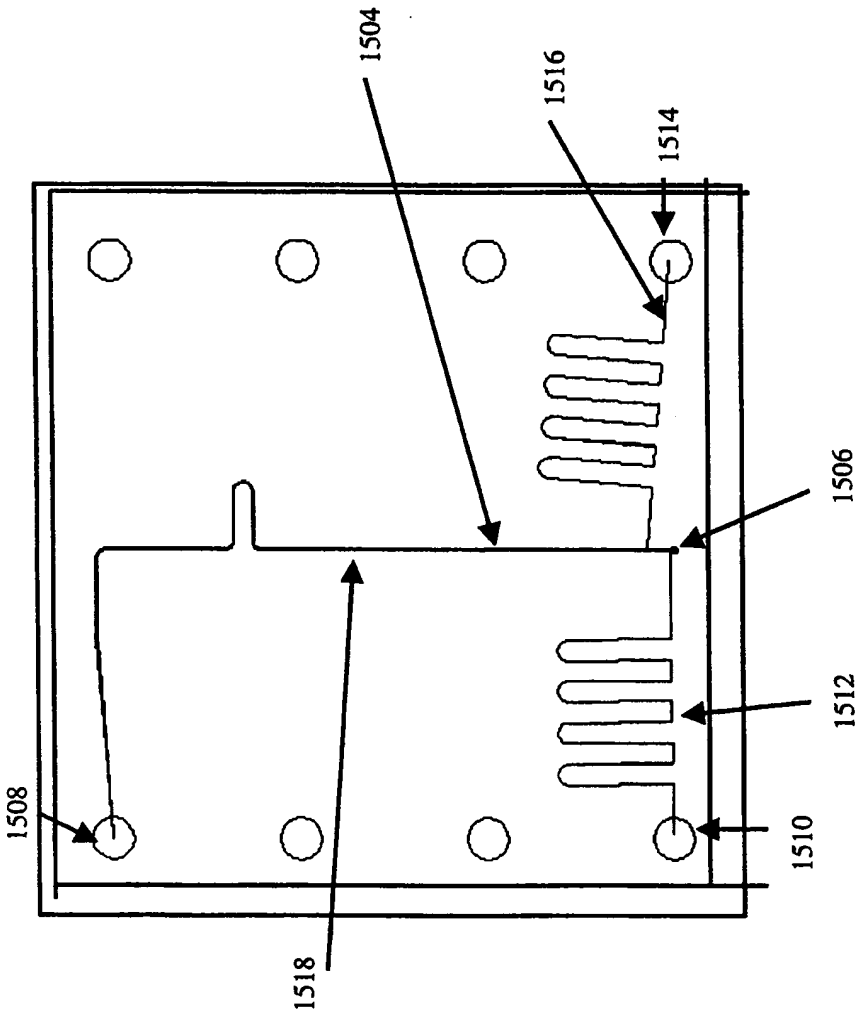

Figure 15

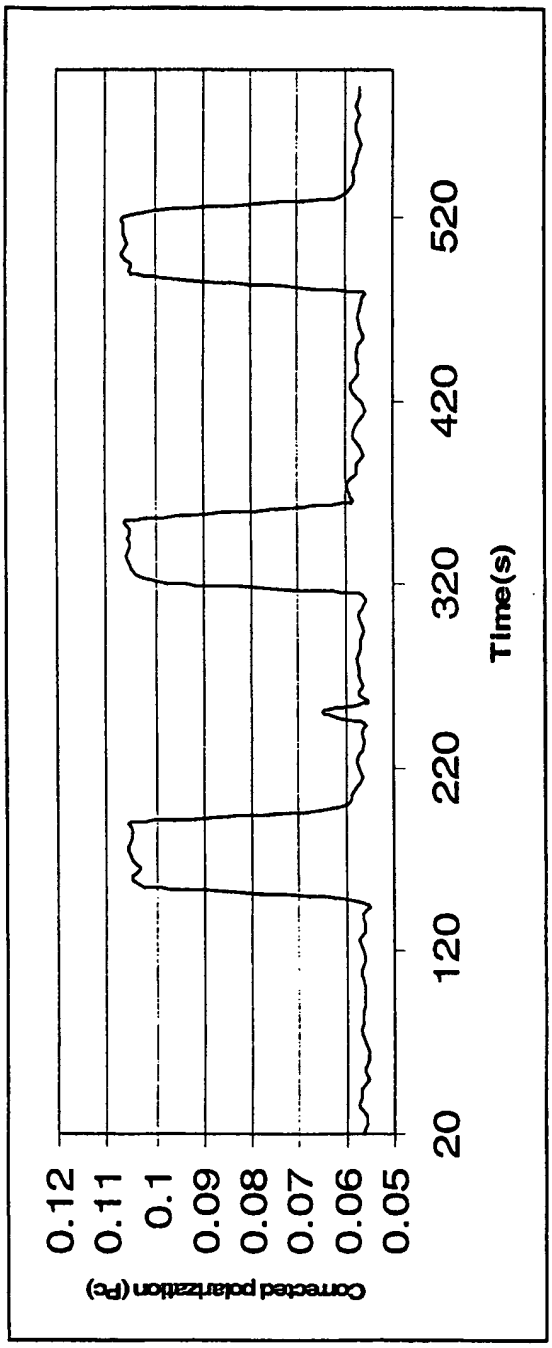
Corrected polarization (Pc)
0.12
0.11
0.1
0.09
0.08
0.07
0.06
0.05
20
120
220
320
420
520
Time(s)

Figure 16

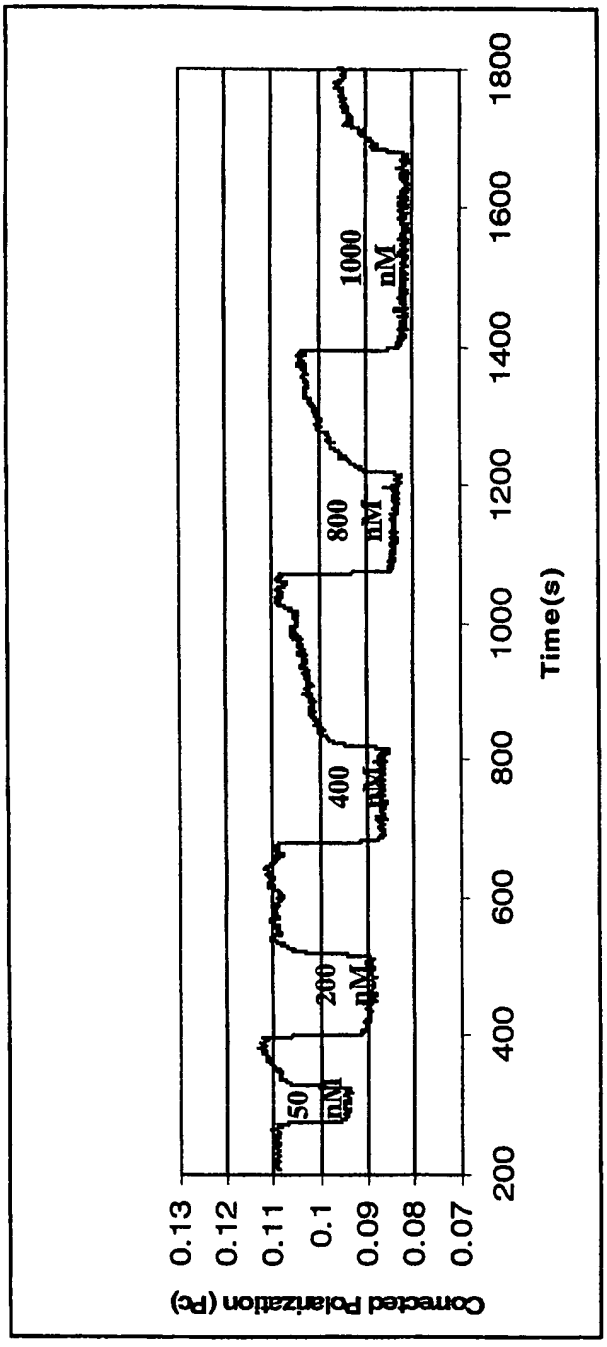
Corrected Polarization (Pc)
0.13
0.12
0.11
0.1
0.09
0.08
0.07
200
400
600
800
1000
1200
1400
1600
1800
Time(s)
50 nM
200 nM
400 nM
800 nM
1000 nM

Figure 17

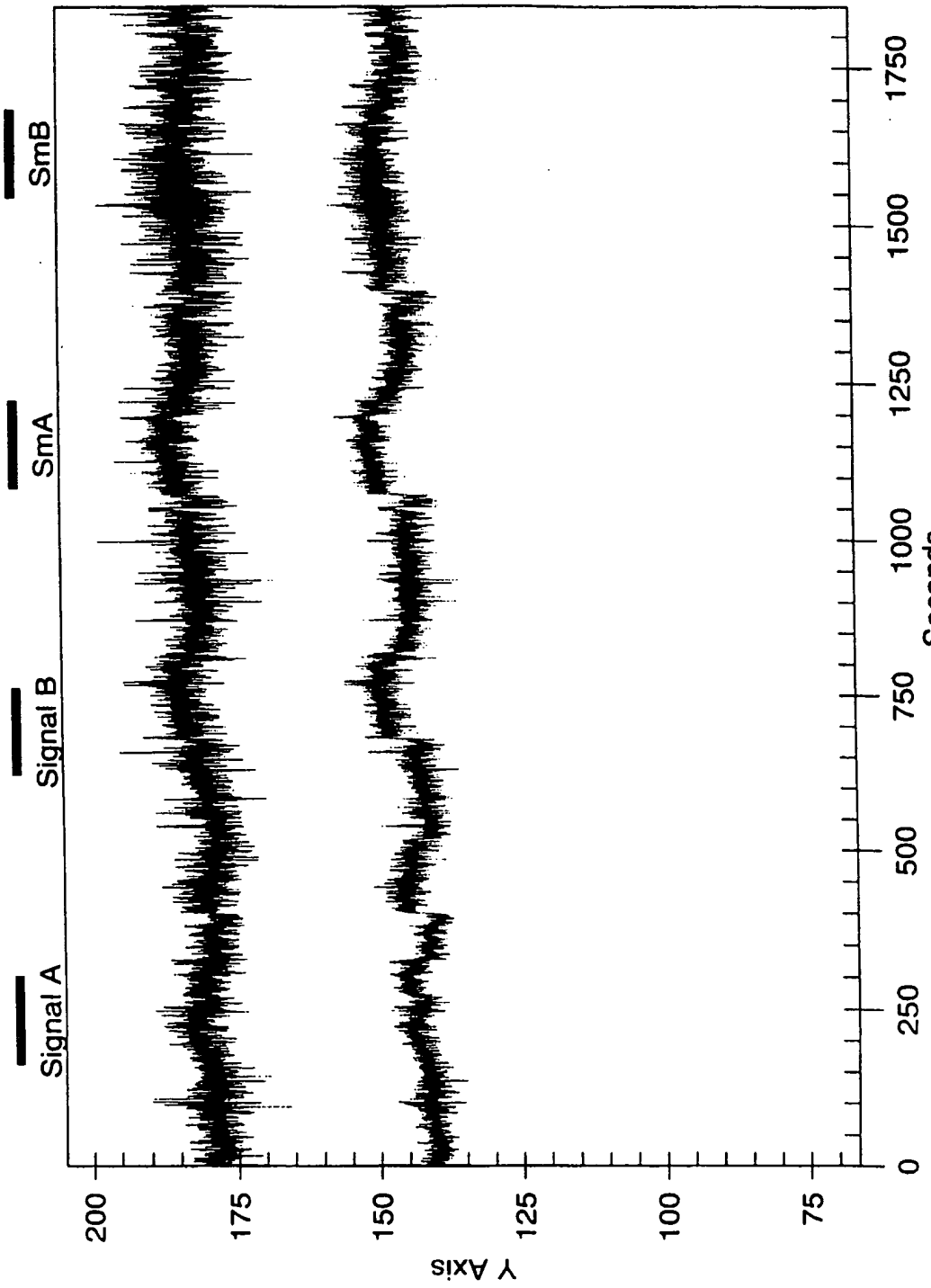
Signal A
Signal B
SmA
SmB
Y Axis
200
175
150
125
100
75
0
250
500
750
1000
1250
1500
1750
Seconds

Figure 18

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/12671

A. CLASSIFICATION OF SUBJECT MATTER

IPC( ) : G01N 15/06, 21/00 and US: 422/55, 68.1; 436/172
US CL : 422/55, 68.1: 436/172
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

: US: 422/55, 68.1: 436/172

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,605,662 A (HELLER et al) 25 February 1997, see col. 5, lines 60-64; col. 6, lines 24-40; col. 20, line 46; and claims, see entire document.	1-24
X	WO 96/04547 A (LOCKHEED MARTIN ENERGY SYSTEMS,INC) 15 February 1996, see abstract, page 4, paragraph	1-6
Y	4, figures and claims, see entire document.	7-24
X	US 4,675,300 A (ZARE et al) 23 June 1987, see figures, abstract and claims, as well as entire document.	12
Y		13-18
Y	WO 96/15576 A1 (DAVID SARNOFF RESEARCH CENTER, INC.C) 23 May 1996, see claims, figures, abstract and entire document.	1-24

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report 01 NOvember 1999 (01.11.99)
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No.	Authorized officer Kelli Collins for Telephone No.

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/12671

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,554,339 A (COZZETTE et al) 10 September 1996, see figure 7a and entire document.	1,3-6,8-13, 15,17
X,P	US 5,709,837 A (MORI et al) 20 January 1998, see abstract, claims 9, 19 and entire document.	1,3,4,6,7
X	US 5,286,624 A (TERASHIMA et al) 15 February 1994, see abstract, col. 3-5, 8-9 and claims.	13,14,16, 18
X	US 5,420,016 A (BOGUSLASKI et al) 30 May 1995, see abstract and entire document.	1,3,5-7